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Purification of chitinase/chitosanase from *Bacillus cereus* and discovery of an enzyme inhibitor

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ABSTRACT

A chitinase and a chitosanase were induced from a squid pen powder (SPP)-containing medium of *Bacillus cereus* TKU030 and purified by precipitation with ammonium sulphate and combined column chromatography. The purified chitinase and chitosanase exhibited optimum activity at 60 °C, pH 5–6 and 40 °C, pH 4, respectively. The chitinase and chitosanase were stable at 25–60 °C, pH 4–7 and 25–50 °C, pH 3–7, respectively. The chitinase and chitosanase showed the highest activity toward β-chitin and 60% DD chitosan, respectively. The chitinase was significantly inhibited by Mn^{2+} and EDTA but activated by Cu^{2+}, Fe^{2+} and Ca^{2+}. The chitosanase was significantly inhibited by Cu^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+} and EDTA. The chitinase showed high stability in the presence of various surfactants, such as SDS, Tween 20, Tween 40 and Triton X-100. In contrast, these surfactants were inhibitors of the chitosanase. The chitinase and chitosanase were also inhibited by TKUPSP017, a small synthetic boron-containing molecule with a BF_3K side-chain. However, TKUPSP017 enhanced the growth of *B. cereus* TKU030 in SPP-containing medium.

1. Introduction

Chitinase and chitosanase have been found in abundance in a variety of bacteria [1–11], and they are capable of catalyzing the hydrolysis of chitin or chitosan into smaller *N*-acetylchitooligosaccharides or chitooligosaccharides [12]. Some *N*-acetylchitooligosaccharides or chitooligosaccharides have been reported to possess various bioactivities [13,14]. However, most of the chitinase- or chitosanase-producing strains use chitin or chitosan as a major carbon source [10,11,15]. Chitin is one of the most widespread biopolymers in nature [16]. Among the natural chitinous resources, fishery wastes (shrimp/crab shells and squid pens) have an especially high content. Indeed, according to Japanese and Chinese fishing data [17], the annual catch of swordtip squid is approximately 10,000 metric tons (mt), with approximately 100 metric tons (mt), or 1%, due to squid pens. The large amount of chitinous waste sometimes becomes an environmental threat due to its accumulation and slow degradation. Furthermore, the price of SPP is very low (about \$0.30 kg^{-1}). The production of inexpensive chitinase and chitosanase is an important factor for the utilization of SPP. Therefore, organisms that produce chitinase or chitosanase with SPP as the sole C/N source cannot only solve an environmental problem but also decrease the production cost of microbial chitinase or chitosanase.

Chitin is also an essential structural component of fungal cell walls [1]. Chitinases degrade the chitin and play key roles in the life cycles of pathogenic fungi [18]. Hence, chitinase inhibitors may serve as potential biocontrol agents. Allosamidin is a known chitinase inhibitor of natural origin, but its use is unfortunately restricted by its limited availability and high cost [18]. Consequently, the development of new antifungal agents has become an absolute necessity for agricultural applications.

This paper describes the purification and characterization of the chitinase and chitosanase, co-induced by SPP from a strain in soil, *B. cereus* TKU030. We further discovered a small synthetic boron-containing molecule (TKUPSP017), which inhibited the chitinase and chitosanase, suggesting that it could be used as a new antifungal agent. Surprisingly, TKUPSP017 promoted the growth of *B. cereus* TKU030.

2. Materials and methods

2.1. Materials

The squid pen powder (SPP) used in these experiments was prepared as described previously [3]. The squid pens were purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). During the preparation of the SPP, the squid pens were washed thoroughly

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with tap water and then dried. The resulting dried materials were milled to powders for use as the carbon source for chitinase and chitosanase production. The Sephacryl S-100 and DEAE-Sepharose CL-6B were purchased from GE healthcare UK Ltd. (Little Chalfont, Buckinghamshire, England). The test enzyme inhibitors (TKUPSP017, TKUPSP018, TKUPSP011, TKUPSP035, TKUPSP074, TKUPSP073, TKUPSP080 and TKUPSP062) were kindly supplied by Dr. P.S. Pan, Department of Chemistry, Tamkang University, New Taipei City, Taiwan. All other reagents used were of the highest grade available.

2.2. Screening and identification of chitinase- and chitosanase-producing strains

The microorganisms isolated from soil samples collected at different locations in northern Taiwan were screened on agar plates containing 1% SPP, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$ and 1.5% agar powder (pH 7). The plates were incubated at 30 °C for 2 days. The organisms obtained from this screening were subcultured in liquid media (containing 1% SPP, 0.1% K_2HPO_4 and 0.05% $MgSO_4{\cdot}7H_2O$) in shaking flasks at 30 °C on a rotary shaker (150 rpm, Yih Der LM-570R). After incubation for 2 days, the culture broth was centrifuged (4 °C at 12,000 × *g* for 20 min, Kubota 5922), and the supernatants were collected for the measurement of chitinase and chitosanase activity using the procedure described below. The TKU030 strain showed the highest chitinase and chitosanase activity, and it was isolated, maintained on SPP agar and used throughout the study.

The bacterial strain TKU030 was identified using morphological, physiological and biochemical parameters as well as a 16S rDNA-based sequence analysis after PCR amplification with specific primers. The nucleotide bases of the DNA sequence obtained were compiled and compared with sequences in the GenBank databases using the BLAST program. Further identification of strain TKU030 was performed using the analytical profile index (API).

Strain TKU030 grew on nutrient agar plates. The bacteria which grew on the surface of the agar plate were suspended by gentle mechanical agitation in 2 mL of sterile distilled water. This bacterial suspension was used to inoculate 50 CHB API strips (ATB system, bioMérieux SA, Marcy-l'Etoile, France) following the manufacturer's instructions. The strips were incubated at 30 °C and observed after 16, 24, 40 and 48 h and compared to the API identification index and database.

2.3. Purification of the chitinase and chitosanase

2.3.1. Production of chitinase and chitosanase

For the production of chitinase and chitosanase, *B. cereus* TKU030 was grown in 50 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 1% SPP, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$ (pH 7). One milliliter of the seed culture was transferred into 50 mL of the same medium and grown in an orbital shaking incubator for 4 days at 37 °C and pH 7.2 (the pH after being autoclaved was 7.5). After incubation, the culture broth was centrifuged (4 °C at 12,000 × *g* for 20 min), and the supernatant was used for further purification via chromatography.

2.3.2. Sephacryl S-100 chromatography

Ammonium sulphate (608 g/L) was added to the culture supernatant (440 mL). The resulting mixture was stored at 4 °C overnight, and the precipitate that had formed was collected by centrifugation at 4 °C for 20 min at 12,000 × *g*. The precipitate was then dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7) and dialysed against the buffer. The resulting dialysate (3 mL) was loaded onto a Sephacryl S-100 gel filtration column (2.5 cm × 100 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7) and eluted with the same buffer. One peak exhibiting chitinase and chitosanase activity for the enzyme solution was obtained. The fractions having peak chitinase and chitosanase activity were pooled and concentrated using ammonium sulphate precipitation. The resultant precipitate was collected by centrifugation and dissolved in 5 mL of 50 mM sodium phosphate buffer (pH 7).

2.3.3. DEAE-Sepharose CL-6B chromatography

The resulting enzyme solution was loaded onto a DEAE-Sepharose CL-6B column (5 cm × 30 cm) that had been equilibrated with 50 mM sodium phosphate buffer (pH 7). One chitinase was washed from the column with the same buffer, and a chitosanase was eluted with a linear gradient of 0–1 M NaCl in the same buffer. The fractions of the two peaks containing the chitinase or chitosanase activity were independently pooled and concentrated using ammonium sulphate precipitation. The pooled fractions for each enzyme solution were used as a purified preparation.

2.4. Protein determination

The protein content was determined using the Bradford method with a Bio-Rad dye reagent concentrate and bovine serum albumin as the standard. After column chromatography, the protein concentration was estimated by measuring the absorbance at 280 nm [3].

2.5. Measurement of chitinase and chitosanase activity

Colloidal chitin (1.3% in 50 mM phosphate buffer) was used as the substrate for the measurement of chitinase activity. A mixture of enzyme solution (0.5 mL) and substrate (1 mL) was incubated at 37 °C for 30 min. After centrifugation, the amount of reducing sugar produced in the supernatant was determined by the method of Imoto and Yagishita [19], with *N*-acetylglucosamine as a reference compound. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars per min.

The chitosanase activity of the enzyme was measured by incubating 0.2 mL of the enzyme solution with 1 mL of 0.3% (w/v) water-soluble chitosan (Kiotec Co., Hsinchu, Taiwan; with 60% deacetylation) in 50 mM phosphate buffer, pH 7, at 37 °C for 30 min. The reaction was stopped by heating the reaction mixture to 100 °C for 15 min. The amount of reducing sugar produced was measured using the method of Imoto and Yagishita [19], with glucosamine as a reference compound. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars per min. The specific activity was expressed as units per mg protein (U/mg protein) of the enzyme extract.

2.6. Determination of molecular mass

The molecular mass of the purified chitosanase was determined using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) [20] with 12.5% acrylamide and 2.67% methylene bis acrylamide in a 0.375 M Tris–HCl buffer (pH 8.8) with 0.1% (w/v) SDS. Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7) containing β-mercaptoethanol. The electrode buffer was 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS (pH 8.3). Electrophoresis was performed at a constant current of 70 mA through the stacking gel and 110 mA through the resolving gel. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 in a methanol–acetic acid–water (5:1:5, v/v) solution and decoloured in 7% acetic acid. The molecular mass of the purified chitosanase in its native form was determined using a gel filtration method. The sample and standard proteins were applied