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Conversion and degradation of shellfish wastes by *Bacillus cereus* TKU018 fermentation for the production of chitosanases and bioactive materials

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ABSTRACT

Two chitosanases (CHSB1 and CHSB2) were purified from the culture supernatant of *Bacillus cereus* TKU018 with shrimp shell as the sole carbon/nitrogen source. The molecular masses of CHSB1 and CHSB2 determined by SDS–PAGE were approximately 44 kDa and 22 kDa, respectively. The optimum pH, optimum temperature, pH stability, and thermal stability of CHSB1 and CHSB2 were (pH 5, 60 °C; pH 5–7, <40 °C) and (pH 7, 50 °C; pH 4–7, <50 °C), respectively. CHSB1 and CHSB2 were both inhibited by EDTA and CHSB1 was inhibited completely by 5 mM Zn^{2+}. CHSB1 and CHSB2 degraded chitosan with DD ranging from 60% to 95%, but did not degrade chitin. The most susceptible substrate was 60% deacetylated chitosan. Furthermore, TKU018 culture supernatant (1.5% SPP) incubated for 3–4 days has 75% relative antioxidant activity (DPPH scavenging ability). With this method, we have shown that shellfish wastes may have a great potential for the production of bioactive materials.

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1. Introduction

Chitosan, a D-glucosamine polymer, is a totally or partially deacetylated derivative of chitin. It is usually obtained by the artificial deacetylation of chitin in the presence of alkali [1]. However, the application of the natural polysaccharides in medicine and food industry is limited since its high molecular weight results in low solubility in acid-free aqueous media. Therefore, recent studies have been focusing on converting chitin and chitosan to oligosaccharides because the oligosaccharides not only are water-soluble but also possess versatile functional properties such as antitumor activity and antimicrobial activity [1–3]. Traditionally, chitosan oligosaccharides were processed by chemical methods in industries. There are many problems existing in chemical processes, such as a large amount of short-chain oligosaccharides produced, low yields of oligosaccharides, high cost in separation, and also environmental pollution. Alternatively, with its advantages in environmental compatibility, low cost, and reproducibility, the use of chitosanase for the hydrolysis of chitin and chitosan has become popular in recent years [4,5].

Chitosanases have been found in abundance in a variety of bacteria, including *Bacillus* spp. [6,7]. Almost all of the chitosanase-producing strains used colloidal chitosan or chitosan as a major carbon source, such as *Bacillus cereus* D-11 [7], *B. cereus* S1 [8] and *Aspergillus* sp. CJ22-326 [9]. However, preparation of chitin/chitosan involves demineralization and deproteinization of shellfish waste by the use of strong acids or bases [1,10]. The utilization of shrimp shell and squid pen wastes not only solves environmental problems but also decreases the production cost of microbial chitosanases. Among these published chitosanase-producing strains, few have been found to utilize marine wastes as carbon/nitrogen source. The production of inexpensive chitosanase is an important element in the process.

The purpose of this study was to isolate chitosanase-producing bacteria, then to purify and characterize the chitosanases from the bacteria, *B. cereus* TKU018, and to compare with chitosanases isolated from other bacterial sources.

2. Materials and methods

2.1. Materials

The shrimp shell powder (SSP) and squid pen powder (SPP) used in these experiments were prepared as described earlier [1]. SSP and SPP were purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). DEAE-Sepharose CL-6B, Phenyl Sepharose and Sephacryl S-100 were purchased from GE Healthcare UK Ltd. (Little Chalfont, Buckinghamshire, England). Weak-base anion-exchanger Macroprep DEAE was from Bio-Rad (Hercules, CA, USA). All other reagents used were of the highest grade available.

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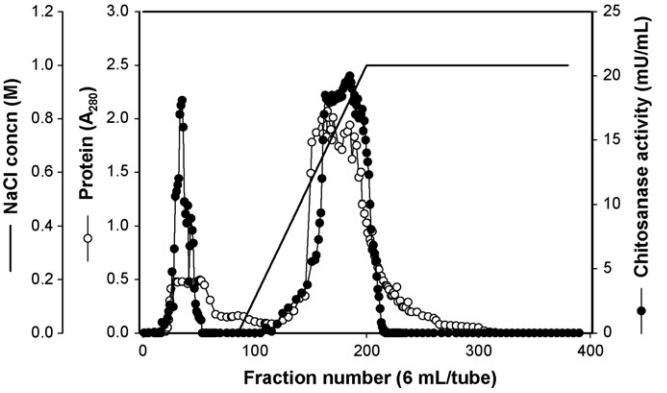

Fig. 1. Elution profile of TKU018 chitosanases on DEAE-Sepharose CL-6B: (○) absorbance at 280 nm; (●) chitosanase activity (mU/mL).

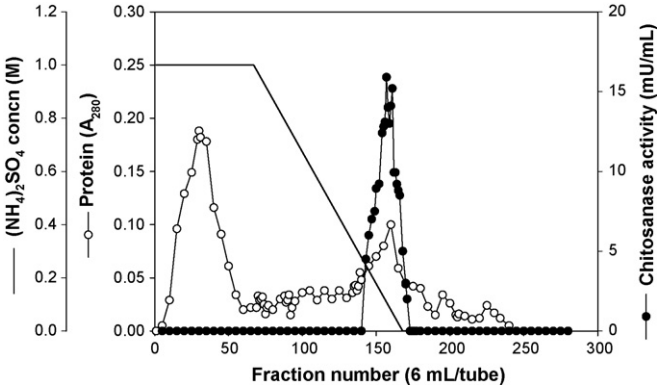

Fig. 2. Elution profile of TKU018 chitosanase (CHSB1) on Phenyl Sepharose 6 Fast Flow: (○) absorbance at 280 nm; (●) chitosanase activity (mU/mL).

2.2. Isolation and screening of chitosanase-producing strains

Microorganisms isolated from soils collected at different locations in northern Taiwan were screened on agar plates containing 1% SSP, 0.1% K_2HPO_4, and 0.05% $MgSO_4{\cdot}7H_2O$, and 1.5% agar powder (pH 7). The plates were incubated at 30 °C for 2 days. Those organisms obtained from the screening were subcultured in liquid media (containing 1% SSP, 0.1% K_2HPO_4, and 0.05% $MgSO_4{\cdot}7H_2O$) in shaking flasks at 30 °C and 150 rpm. After incubation for 2 days, the culture broth was centrifuged (4 °C and 12,000 × *g* for 20 min) and the supernatants were collected for measurement of chitosanase activity using the procedure described below. The strain TKU018 that showed the highest chitosanase activity was isolated, maintained on nutrient agar, and used throughout the study.

2.3. Purification of the chitosanases

2.3.1. Production of chitosanases

For the production of chitosanases, *B. cereus* TKU018 was grown in 100 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 0.5% SPP, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$ (pH 7). One milliliter of the seed culture was transferred into 100 mL of the same medium and grown in an orbital shaking incubator for 3 days at 30 °C and pH 7 (the pH after being autoclaved was 7.5). After incubation, the culture broth was centrifuged (4 °C and 12,000 ×*g* for 20 min), and the supernatant was used for further purification by chromatography.

2.3.2. DEAE-Sepharose CL-6B chromatography

To the culture supernatant (900 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 12,000 × *g*. The precipitate was then dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7), and dialyzed against the buffer. The resultant dialysate (50 mL) was loaded onto a DEAE-Sepharose CL-6B column (5 cm × 30 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7). As shown in Fig. 1, one chitosanase (CHSB1) was washed from the column with the same buffer and another chitosanase (CHSB2) was eluted with a linear gradient of 0–1 M NaCl in the same buffer. The fractions of the two peaks containing the chitosanase activity were respectively pooled and concentrated by ammonium sulfate precipitation. The resultant precipitates were collected by centrifugation and dissolved in 5 mL of 50 mM sodium phosphate buffer (pH 7).

2.3.3. Phenyl Sepharose chromatography

The obtained enzyme solution (the unadsorbed chitosanase fractions from DEAE-Sepharose CL-6B column) was then chromatographed on a column of Phenyl Sepharose (1.3 cm × 20 cm), which had been equilibrated with 50 mM sodium phosphate buffer (pH 7) containing 1 M $(NH_4)_2SO_4$. The chitosanase was eluted with a linear gradient of 1–0 M $(NH_4)_2SO_4$ in the same buffer. As shown in Fig. 2, the chitosanase fractions were collected and the enzyme activity was measured. Fractions with confirmed enzyme activity were pooled, dialyzed overnight at 4 °C against 50 mM sodium phosphate buffer pH 7, and lyophilized.

2.3.4. Macro-prep DEAE chromatography

The obtained enzyme solution (the adsorbed chitosanase fractions from DEAE-Sepharose CL-6B column) was then chromatographed on a column of Macro-prep DEAE (12.6 mm × 40 mm), which had been equilibrated with 50 mM sodium phosphate buffer (pH 7). As shown in Fig. 3, the chitosanase was eluted with a linear gradient of 0–1 M NaCl in the same buffer. The fractions containing the chitosanase activity (Fig. 3) were pooled and concentrated by ammonium sulfate precipitation. The resultant precipitate was collected by centrifugation and dissolved in 50 mM sodium phosphate buffer (pH 7).

2.3.5. Sephacryl S-100 chromatography

These two resultant enzyme solutions were respectively loaded onto a Sephacryl S-100 gel filtration column (2.5 cm × 120 cm), which had been equilibrated with 50 mM sodium phosphate buffer (pH 7), then eluted with the same buffer. One peak exhibiting chitosanase activity for each enzyme solution was obtained and pooled fractions for each enzyme solution were used as a purified preparation.

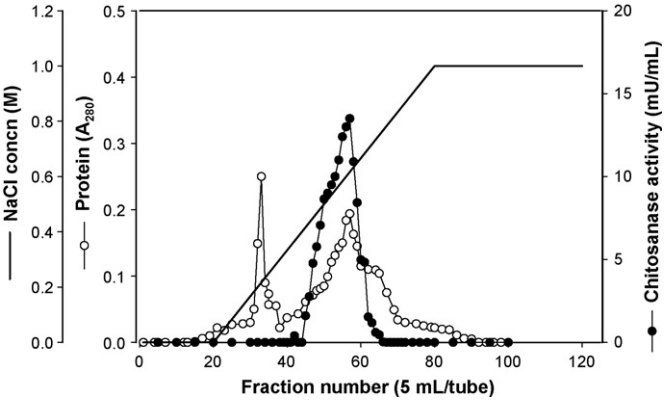

Fig. 3. Elution profile of TKU018 chitosanase (CHSB2) on Macro-prep DEAE: (○) absorbance at 280 nm; (●) chitosanase activity (mU/mL).

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