Journal of Bioscience and Bioengineering
VOL. 117 No. 6, 684–689, 2014

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Characterization of an alkalophilic extracellular chitosanase from *Bacillus cereus* GU-02

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Received 20 August 2013; accepted 4 November 2013
Available online 9 December 2013

An alkalophilic extracellular chitosanase (ACTase) was characterized from the culture supernatant of *Bacillus cereus* GU-02. Kinetic properties of ACTase produced from *B. cereus* GU-02 after cultivation in anaerobic condition, alkaline medium (pH 10) at 37°C for 3 days were investigated. ACTase was found to be stable in alkaline pH range from 8 to 10. Interestingly, optimum pH and temperature were estimated to be 10 and 37°C, respectively, where ACTase showed chitosan degrading activity (87%), which was enhanced by 15% in the presence of calcium ions (8 mM). The ACTase produced from *B. cereus* GU-02 was partially purified from the culture supernatant, and its enzymatic activity was kinetically characterized. The V_{max} and K_m were estimated with a chitosan (degree of deacetylation, DD 92% as substrate) as 0.038 U/min/μg protein and 0.327 μM, respectively. A combination of the TLC and MALDI-TOF MS results showed that the chitosan oligosaccharides obtained from the hydrolysis of high molecular weight chitosan (HMWC) by ACTase of the *B. cereus* GU-2 comprise oligomers with degree of polymerization (DP) mainly from dimers to pentamers. High production of ACTase and chitooligosaccharides may be useful for various industrial and biological applications.

[**Key words:** *Bacillus cereus*; Alkalophilic chitosanase; Chitosan; Chitooligosaccharides; Kinetic properties]

Chitin, a β-1,4-linked linear homopolymer of *N*-acetylglucosamine, is found in the exoskeletons of insects, crustaceans and cell walls of certain fungi (1) and known as the second most abundant polysaccharide after cellulose. Full or partial deacetylation of chitin produces chitosan, a linear polysaccharide composed mainly β-1,4-2-deoxy-2-amino-D-glucopyranose (2). Several studies have previously attracted interest in converting chitosan into chitosan-oligosaccahrides due to high water-solubility and versatile biological properties of chitosan-oligosaccharides (3–6). Enzymatic depolymerizing of chitosan is very useful for producing chitosan-oligosaccharide including di-, tri-, and tetra-oligosacharide mainly. Bacteria producing chitosanase were mostly separated from nature and these chitosanase have similar action patterns to chitosan (7–12).

However, most of chitosanases purified from chitosanolytic enzyme producing bacteria such as *Streptomyces roseolus* (13), *Bacillus subtilis* HD145 (14) and *Acinetobacter calcoaceticus* TKU024 (15) have optimum pH of about 5–6, weak acidic condition, in addition to being unstable under extreme acidic or alkaline condition thus limiting their application, bioconversion and utilization. Therefore, screening of new chitosanlytic enzyme with stability in the extreme acidic or alkaline condition from soil and marine environment is required for extending application and utilization of chitosanase in various fields.

Chitin and chitosan are widely distributed in living organisms such as crustaceans, insects and fungi (1). In each organism, chitinase and chitosanase play important role in biological mechanism essential for their life including aggressive and defensive action toward target, morphogenesis and molting (2). In particular, most chitosanase have been found in various bacteria and fungi (3–8). Additionally, these chitosanase are useful for the preparation of chitosan oligosaccharide with biofunctionality. Such chitosan oligosaccharides are expected to be subjected for medical components and functional food due to their beneficial physiological activities including antitumor activity (9,10), antibacterial activity (11,12), immunoenhancing effect (16) and anti inflammatory effect (17).

In addition, such functional properties of chitosan oligosaccharide are strongly dependent upon its molecular weight. Pentamers and hexamers are particularly active. However, enzymatic digestion of chitosan does not result in a high content of pentamer or hexamer because they are intermediate products. Also most chitosanases from the various source reported so far were mainly *endo*-acting enzyme in nature, releasing mixture of glucosamine dimers, trimers and tetramers predominantly (18–21). Therefore, screening of new chitosanolytic enzyme capable of producing glucosamine pentamers and hexamers from chitosan should be required to produce these oligomeric chitosan more efficiently.

In an attempt to screen chitosanolytic enzyme which are capable of converting chitosan into big size-oligomeric chitosan, new bacterial strain with chitosan degrading activity was sought. A *Bacillus cereus* strain that was capable of degrading chitosan rapidly was isolated from soil samples. The enzyme responsible for producing big size-oligomeric chitosan was partially purified and characterized, enzymatically. The chitosanase was active over a

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2013.11.005

wide range of pHs and particularly possessed a higher catalytic activity in alkaline side than that of chitosanases isolated previously. This study describes the purification and characterization of an alkalophilic extracellular chitosanase (ACTase), from a culture filtrate of bacterial strain *B. cereus* GU-02 (KCTC 12381BP). This enzyme may be a useful tool for the industrial production of chitooligosaccharides $(GlcN)_n$, $n = 2-5$ and for research on the biological functions of $(GlcN)_n$ in various applications.

MATERIALS AND METHODS

Materials Powdered chitosan (*N*-deacetylated 92%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used for the measurement of chitosanase activity. Alginate was purchased from Samchun Chemical Co., Ltd. (Gyeonggido, Korea), and fucoidan was purchased from Sigma Chemical Co. Silica gel 60/Kieselguhr F_{254} thin-layer chromatography (TLC) plates were purchased from Sigma Chemical Co. and ELISA (Quant, BIO-TEK instruments, Inc., Winooski, VT, USA) was used for measurement of absorbance. Unless stated otherwise, all of the chemicals were of analytical grade.

Bacterial strain and culture condition Bacteria were collected from compost and soil samples in InCheon-city in South Korea and screened for chitosan degrading activity after initial cell growth in basal liquid medium (pH 8.6) consisting of 1% starch, 0.05% $MgSO_4$, 0.01% Na_2HPO_4, 0.5% $NaCO_3$. 0.2 g of soil sample was suspended in dH_2O and the soil was eliminated by centrifugation gently, and the supernatant was centrifuged to harvest the suspended cells. The cell pellet harvested was washed three times, suspended in saline buffer, and spread on agar plate containing complete medium to obtain the various heterotrophic bacteria as well as on basal agar medium containing 2 % powdered chitosan that was selective for chitosanase activity (22). Colonies having chitosan hydrolyzing activity were purified by repeated alternate amplification and selection.

Preparation of chitosanase The chitosanolytic enzyme in the 1 L cell free culture filtrate of *B. cereus* GU-02 in 25 mM Tris–HCl buffer (pH 7.5) was precipitated with ammonium sulfate at 40% saturation and then centrifuged at 13,500 rpm for 30 min to precipitate enzyme and dissolved in the same buffer, and dialyzed (10 kDa MWCO) extensively against 2 L of the same buffer for 24 h at 4°C. The dialyzed enzyme solution (15 ml) was separated and concentrated by using 10-kDa and 50 kDa of molecular weight cut off membranes, respectively. Active enzyme was then pooled together (6 ml) and precipitated with ammonium sulfate at 60% saturation. After centrifugation, precipitated proteins were further dialyzed again the buffer as described above. Proteins were then applied to a column of Superdex 75 10/300 GL chromatography to evaluate the purity and to determine the size of enzyme in native condition. In addition, the molecular weight of the purified enzyme was determined by SDS-PAGE analysis and the purity was further analyzed by following the protocol using a silver staining kit (ELPIS Biotech Inc., Taejon, Korea), using a pre-stained pro-stain ladder ranging from 10 to 170 kDa (Fermentas, USA). Concentration of the purified enzyme was determined according to the method of Bradford using BSA as standard. Subsequently, the substrate specificity was determined using various polysaccharides such as alginate, beta-glucan and high molecular chitosan.

Chitosanase assay Chitosan degrading activity was assayed as follows. Each reaction mixture contained 1 ml of Tris–HCl buffer (50 mM, pH 8.5), 1 ml of a 1 % (w/v) chitosan solution, and 5 μg/ml of enzyme solution. After incubation at 37°C for 1 h, the reaction was stopped by heating at 100°C for 10 min, followed by cooling down the reaction mixture at room temperature for 10 min. Denatured protein was eliminated by centrifugation and the concentration of reducing sugars released during hydrolysis of chitosan was measured using 4-hydroxy-benzhydrazid (PAH-BAH) reagent (23). Glucosamine was used as standard material. One unit of activity was defined as the amount of enzyme that liberated 1 μmol equivalent reducing sugar in 1 min at 37°C.

Optimum reaction condition and stability In order to measure the optimum temperature, the enzyme reaction was carried out at a wide range temperature from 20°C to 50°C for 1 h in Tris–HCl buffer, pH 8. For measurement of the optimum pH, the enzyme activity was estimated at different pHs from pH 3 to 6 using 100 mM acetate buffer, pH 6 to 8 using 100 mM potassium phosphate buffer, pH 8 to 10 using 100 mM sodium borate and pH 10 to 11 using 100 mM sodium carbonate, respectively. For determination of thermostability, the enzyme was initially pre-incubated at various temperature 27–50°C for 2 h under standard condition without the substrate, following which retained activity was measured with various substrates at optimum temperature (Table 1). Also, the pH stability of the enzyme was determined after pre-incubation at various pHs 3 to 11 without the substrate for 2 h, after which retained activity was measured at standard condition, respectively. For determination of effect of metal ions on enzyme activity, the enzyme was incubated with 0.5 mM metal ions dissolved in the buffer and the enzyme activities were determined under optimum condition.

Kinetic characterization To determine the K_m and V_{max}, kinetics characteristics of the enzyme using chitosan were estimated. The initial reaction rate of the enzyme was measured at different chitosan concentration ranging from 0% to 0.5% (w/v) dissolved in 100 mM sodium potassium phosphate buffer, pH 9. The values of K_m and V_{max} were estimated by plotting Woolf–Augustinsson–Hofstee plot (21). One unit (U) of the enzyme activity was defined as the amount of enzyme required to produce the increase of 0.28 in concentration of reducing sugar per minute. The reaction mixtures were terminated by heating at 100°C for 10 min, cooled down at room temperature for 10 min, and centrifuged to remove denatured protein. The reaction products were analyzed by thin-layer chromatography with Merck TLC silica gel G-25 and the solvent system consisting of *n*-propanol: 28% ammonia: water (70/15/15, v/v) was used and visualized by heating 180°C for 10 min after immersing in solution consisting of 8% phosphoric acid and 3% cupric acetate until the compounds charred and the spots were visible.

TABLE 1. Evaluation of substrate specificity of ACTase from *Bacillus cereus* GU-02.

Substrate	Relative activity (%)
Chitosan	100
Alginate	12.7
β-Glucan	32.7
Fucoidan	16.9
Hyaluronic acid	26.7
Sulfate chitin	9.6

The reaction was carried out in reaction mixture containing 50 μg ACTase and 0.25% of each polysaccharide at 37°C for 18 h in CO_2 incubator. Enzyme activity was determined by measuring the concentration of reducing sugar derived from the degradation of each polysaccharide by crude enzyme from *B. cereus* GU-02.

Statistical analysis Data obtained from three separate experiments were expressed as the means of ±SD of activity, unless indicated as the means of averages of experiments. The significance of the difference between averages of experiments and mean values in each experiment was statistically determined by using package of GraphPad InStat (GraphPad InStat Version 3.00, GraphPad Software, Inc., La Jolla, CA, USA). *P*-values less than 0.05 were considered.

RESULTS AND DISCUSSION

Isolation of chitosanolytic enzyme from *B. cereus* Bacterial strain tentatively designated as GU-02 producing gamma-cyclodextrin (CD) in the presence of starch was isolated originally, and investigated to estimate enzyme activity CGTase (cyclo-glycosyltransferase) on depolymerizing starch. A single colony selected by their capacity to produce gamma-CD was identified as *B. cereus* species, based on 16S rRNA sequence analysis, RT-PCR and

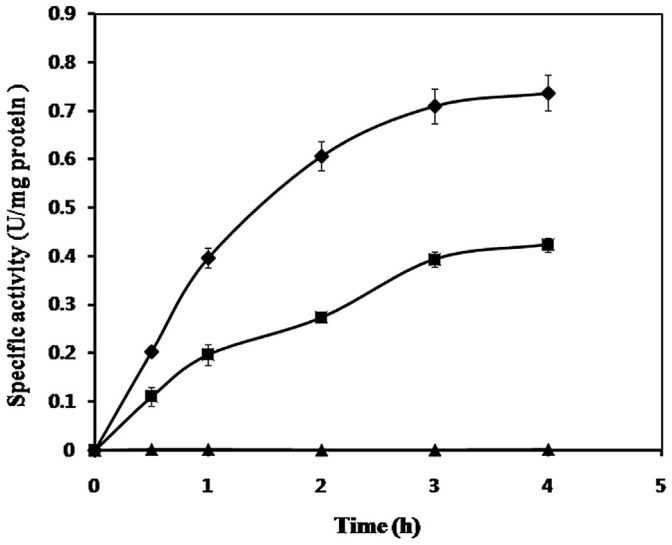

FIG. 1. The assay was performed in the reaction mixture consisting of 0.25% chitosan and 50 μg crude enzyme at 37°C for up to 4 h, respectively. The enzyme activity was determined by monitoring concentration of reducing sugar derived from the degradation of chitosan by each enzyme (closed diamond: enzyme induced by chitosan, closed square: enzyme in pH 8 medium, closed triangle: enzyme in pH 10 medium).