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High degree polymerized chitooligosaccharides synthesis by chitosanase in the bulk aqueous system and reversed micellar microreactors

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Abstract

The chitosanase of *Bacillus cereus* NTU-FC-4 was used for the production of high degree polymerized (DP) chitooligosaccharides. In the aqueous system, the chitosanase was found to be able to catalyze a reaction to yield small amount of octamer from a mixture consisting of dimer, trimer, tetramer, pentamer, hexamer and heptamer, possibly through the transglycosylation reaction. To enhance the possible transglycosylation reaction, the process was carried out in the reversed micellar microreactors formed by AOT (sodium bis-2-(ethylhexyl) sulfosuccinate) in isooctane, and the formation of high DP chitooligosaccharides was significantly increased. It was found that the water content in the reverse micelles was an important factor affecting the enzymatic reaction. When the molar ratio of water to surfactant (W_0) was 11.86, the reaction yielded the highest amount of heptamer, octamer, and nonamer. Hydrolysis of pure chitooligosaccharides by the chitosanase revealed that pentamer and hexamer might be the main gylcosyl acceptors during enzymatic reaction.

Keywords: *Bacillus cereus*; Chitosanase; Chitooligosaccharides; Enzymatic reaction; Reverse micelles; Microreactor

1. Introduction

Chitooligosaccharides possess various bioactive functions, including immunostimulating, antitumor and antibacterial activities [1–3]. Particularly, chitooligosaccharides with the degree of polymerization (DP) equal to or greater than 6 have greater biological activities [4,5].

The chitooligosaccharides could be prepared by chemical or enzymatic hydrolysis of chitin or chitosan. Acids such as hydrofluoric acid [6], hydrochloric acid [7,8], phosphoric acid [9], and nitrous acid [10,11] have been used to hydrolyze chitin or chitosan. However, acid hydrolysis is practically limited by several drawbacks, such as acid corrosion, the need of deacidification after reaction, and the low yield of high DP chitooligosaccharides. Unlike acid hydrolysis, chitosanolytic hydrolysis is more environmental friendly but still produces mixtures of lower DP chitooligomers (from dimer to pentamer) whereas the yield of higher DP chitooligomers (from hexamer to octamer) is relatively low [12].

To increase the yield of higher DP chitooligosaccharides, a reverse approach, using hydrolytic enzyme for synthesis, was applied. The hydrolysis reaction is usually faster than the transglycosylation in the aqueous system [13,14]. However, in theory, the transglycosylation reaction could be enhanced by increasing the substrate concentration, by lowering the water content to modify the hydrolysis reaction, and by precipitating products to increase the rate of reverse hydrolysis. Since the efficiency of transglycosylation is dependent on the ability of acceptor glycoside to compete with water for the enzyme-bound glycon, it is advantageous to carry out the reaction at high concentrations of substrates with a large acceptor to donor ratio [15].

Reverse micelles can be used as microreactors for enzymatic reaction due to the low-water content (i.e. high substrate concentration), amphiphilic characteristic, and the small size to increase the chance of mass transfer [16]. Reverse micelles are spontaneously formed in an organic solvent when certain surfactant molecules and a small amount of water are present

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[17]. The enzyme can be solubilized in the inner core of reverse micelles and maintain its catalytic activity. The reversed micellar system has been successfully applied to synthesize peptides using alpha-chymotrypsin [18], esters using lipase [19], and galatooligosaccharides using β-galactosidase [20].

The objective of this study was to investigate the enzymatic reaction of chitosanase in the reversed micellar microreactors in order to form high DP oligomers. More specifically, factors affecting enzymatic reactions in reversed micellar system were investigated, and attempt has been made to establish the optimal conditions for the enzymatic reaction by chitosanase in the reverse micelles. In addition, the possible mechanism of the enzymatic reaction was also investigated.

2. Materials and methods

2.1. Materials

Chitooligosaccharide standards (dimer to hexamer) were purchased from Seikagaku Co. (Japan). Chitosan was purchased from Lytone Enterprise Inc. (Taipei, Taiwan). The deacetylation degree of the chitosan was 95%, as measured by the colloid titration method [21]. AOT (Sodium bis-2-(ethylhexyl) sulfosuccinate) was a product of Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of analytical grade.

2.2. Preparation of chitosanase

The cultivation of *Bacillus cereus* NTU-FC-4, a microbe isolated from Taiwan soil, was prepared at 30 °C for 2 days in a 500-mL flask containing 200 mL medium, consisting of 0.3% colloidal chitin, 0.5% yeast extract, 0.5% soytone, 0.1% sodium dihydrogen phosphate and 0.05% magnesium sulfate. The culture medium was centrifuged at 4 °C and 6200 rpm for 30 min, then, the supernatant was treated with 70% (v/v) acetone, and centrifuged for 10 min at 7000 rpm. The precipitate was collected and dried by lyophilization [22], and it was used as crude enzyme in this study. One unit of activity of chitosanase was defined as the amount of enzyme needed for hydrolyzing 1% (w/v) chitosan solution to produce 1 μmol reducing sugar/min at 45 °C.

2.3. Preparation of chitooligomer mixture

Chitosan solution was prepared by dispersing chitosan in 0.1 M acetate buffer solution at pH 5.0 and hydrolyzed by chitosanase in a membrane reactor equipped with MWCO 3000 Da hollow-fiber ultrafiltration membrane module (UFP-3-C-4A, AG Technology, MA). The reaction permeate was collected and concentrated to one-twentieth of its original volume with a rotary evaporator and it was used as the substrate of enzymatic reaction. Detailed membrane processing procedure is described elsewhere [22].

2.4. Enzymatic reaction in bulk aqueous system

An amount of 135 unit of crude chitosanase was dissolved in 5 mL of acetate buffer solution (50 mM, pH 5) and mixed with 15 mL of 150 mg/mL substrate solution to obtain a reaction mixture with E/S ratio 0.06 unit/mg. The mixture was incubated at 40 °C in a reciprocating shaker bath (100 rpm). At various time intervals, samples (0.1 mL) were taken, diluted with deionized water (0.4 mL), and then centrifuged for 10 min using NANOSEP (Pall Gelman Sci., MI, USA) micro-concentrator, which was employed to remove the enzyme and to stop the enzymatic reaction. The concentrations of chitooligomers in the filtrate were analyzed by HPLC.

2.5. Reversed micellar extraction of chitosanase

The aqueous solutions were prepared by dissolving an appropriate amount of the freeze-dried enzyme in 50 mM of sodium acetate buffers at pH 5.0. Sodium chloride was added to the aqueous solution to adjust the ionic strength. The organic solution was prepared by dissolving a designated amount of AOT in isooctane. For the forward extraction (i.e. inclusion of enzyme in the reversed micelles), 0.4 mL aqueous solution was injected into 5 mL organic solution in a centrifugal tube (15 mL). The mixture was shaken at 200 rpm in a reciprocating shaker bath at designated temperature for various time periods. For the backward extraction, equal volumes (ca. 4 mL) of the mixture from forward extraction and 50 mM phosphate solution at pH 10.0 containing 1 M KCl were mixed in a centrifugal tube. The mixture was held at 40 °C in a water bath for 5 min, then shaken at 150 rpm for 40 min, and centrifuged at 1075 × *g* for 5 min to separate the two phases. Samples of aqueous phase were then taken for analysis of enzyme activity.

2.6. Enzymatic reaction in reverse micelles

Reverse micelles were prepared by injecting various amounts (5, 8, and 10 mL) of mixture of enzyme and substrate (E/S ratio 0.06 unit/mg) solution into 110 mL AOT in isooctane solution (20% (w/w) AOT in isooctane) at 20 °C to form reverse micelles with various water contents (W_0), which were 7.41, 11.86, and 14.82, respectively. The amount of water in the reverse micelles (W_0) was expressed as the molar ratio of water to the surfactant (AOT) in the reversed micellar phase ($W_0 = [H_2O]/[AOT]$). The concentration of enzyme was 45 mg/mL (90 mg of enzyme was dissolved in 2 mL of 50 mM acetate buffer which contained 200 mM NaCl). The enzymatic reaction was then carried out in a reciprocating shaker bath at 40 °C and 150 rpm. At different time intervals, 8 mL sample was withdrawn, added to 120 mL acetonitrile and vigorously stirred to destabilize the reverse micelle and to release the solubilized compounds. After centrifuged at 9000 rpm for 10 min, the white precipitate was washed out by 10 mL isooctane and centrifuged again. The centrifuged residue was dissolved in deionized water and analyzed by HPLC to determine the composition of chitooligomers.

2.7. Analytical methods

The protein concentration was determined by Lowry method using Bio-Rad protein D_c protein assay kit. SDS-polyacrylamide gel electrophoresis using 10% acrylamide was performed and stained by Coomassie blue-R250 [23]. Chitosanase activity was determined by measuring the reducing sugar produced from chitosan after reaction. Chitosan was dissolved in the 0.2 M acetate buffer at pH 5 to make a 1% (w/v) chitosan solution. A mixture consisting of 1 mL of 1% (w/v) chitosan solution, 3.5 mL of 0.2 M acetic acid solutions and 0.5 mL of enzyme solution was then prepared and incubated at 45 °C for 30 min, then boiled for 15 min to stop the reaction. A portion of the mixture (0.5 mL) was mixed with 1.8 mL of water and 2 mL of alkaline ferri-cyanide solution, and the reducing sugar produced was measured colorimetrically using a standard curve constructed by pure compound of glucosamine [24]. One enzyme unit was defined as the amount of enzyme that hydrolyzed 1% (w/v) chitosan solution to yield 1 μmol of reducing sugar/min at 45 °C.

The hydrolyzed chitooligosaccharide mixture was subjected to thin-layer chromatography (TLC) using silica gel plate [25]. The chitooligosaccharide mixture was spotted onto the TLC plate and developed in propanol/water/ammonia (70:30:1, v/v). Chitooligosaccharide standards (glucosamine, chitobiose, chitotriose, chitotetraose, chitopentaose, and chitohexaose) were run in parallel to the chitooligosaccharide mixture. After solvent development, plate was dried by hot air and immersed in a saturated silver nitrate solution (0.7 g/200 mL acetone). Plate was dried by hot air again and chitooligosaccharides were detected by spraying plate with 0.5N sodium hydroxide/ethanol solution then charring at 80 °C for 20 min.

An ICI HPLC system (LC1100 pump, Australia) equipped with RI detector was used for analyzing chitooligosaccharides content. The chitooligosaccharides were separated on a HYPERSIL HS APS column (25 cm × 4.6 mm, Thermo Instrument Systems Inc., Runcorn, UK), eluted by the mixture of acetonitrile and distilled water (60/40) with a flow rate of 0.8 mL/min at 40 °C. The retention time and concentration of each individual peak in the sample was compared to the peak of standard chitooligosaccharides with DP 1–6. However, the pure compounds of heptamer to nonamer were not available, therefore, the higher DP chitooligomers were identified by the sequence of peaks appeared in the HPLC chromatogram, and the concentrations of these chitooligomers were estimated based on the standard curve of hexamer.