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Process Biochemistry 43 (2008) 76–82

Process Biochemistry

www.elsevier.com/locate/procbio

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

Yi-Chien Hsiao [a], Yu-Wei Lin [a], Chia-Kai Su [b], Been-Huang Chiang [a,*]

[a] *Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan, ROC*

[b] *Department of Leisure, Recreation, and Tourism Management, Southern Taiwan University of Technology, Tainan, Taiwan, ROC*

Received 18 June 2006; received in revised form 4 August 2007; accepted 24 October 2007

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

* Corresponding author at: Institute of Food Science and Technology, National Taiwan University, P.O. Box 23-14, Taipei 10672,Taiwan, ROC. Tel.: +886 2 33664120; fax: +886 2 23620849.

E-mail address: bhchiang@ntu.edu.tw (B.-H. Chiang).

1359-5113/$ – see front matter
doi:10.1016/j.procbio.2007.10.017

[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.

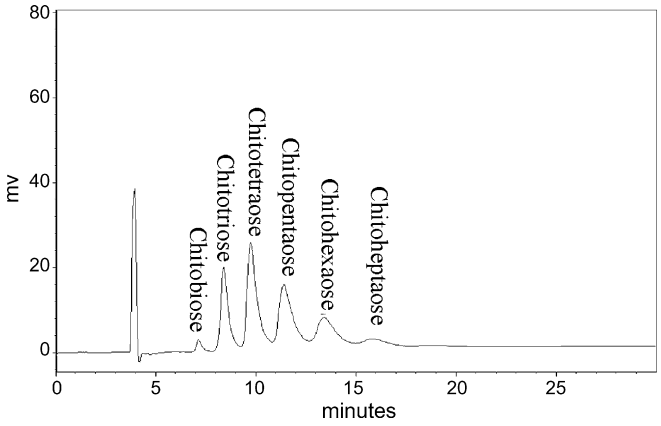

Fig. 1. High-performance liquid chromatogram of chitooligomer mixture.

3. Results and discussion

3.1. Chitooligosaccharides profile in the substrate

The mixture of chitooligomers resulting from the membrane reactor was used as substrate in this study. A typical HPLC profile of the substrate is given in Fig. 1. Because chitosan has to be dissolved in acetic acid and chitosanase has optimal activity at pH 5.0, the acetic acid buffer at pH 5 was used throughout the experimentations. However, during HPLC analysis the retention time of glucosamine was 6 min, which was very close to that of sodium acetate in the buffer. As the result, glucosamine could not be clearly identified and quantitated by HPLC analysis. In order to determine the content of glucosamine in the substrate, TLC analysis was employed and the result is shown in Fig. 2. It appeared that when applying the same concentration of standard solutions of chitooligomers, glucosamine was the most visible one shown in the chromatogram (Fig. 2). However, hardly any spot was visible corresponding to the position of glucosamine developed by the mixture of chitooligomers. Therefore, it could be concluded that the glucosamine was not a main component in the prepared substrate for further enzymatic reaction. Based on the result of HPLC analysis, the substrate was composed of dimer (2.4%, w/w), trimer (27.5%, w/w), tetramer (33.4%, w/w), pentamer (24.8%, w/w), hexamer (10.3%, w/w) and heptamer (1.6%, w/w).

3.2. Enzymatic reaction in bulk aqueous system

Enzymatic reaction using chitosanase and substrate consisting of dimer to heptamer with E/S ratio 0.06 unit/mg in bulk aqueous system was investigated, and the result is given in Fig. 3. The dimer and trimer increased during reaction, and there was no change in tetramer. Pentamer and hexamer decreased as reaction time increased. Octamer was formed after 2 h but both of heptamer and octamer disappeared after 7 h. Nonamer could not be detected. In order to understand the possible mechanism of this enzymatic reaction, pure chitooligomers were used as substrates for chitosanase. Results showed that the chitosanase

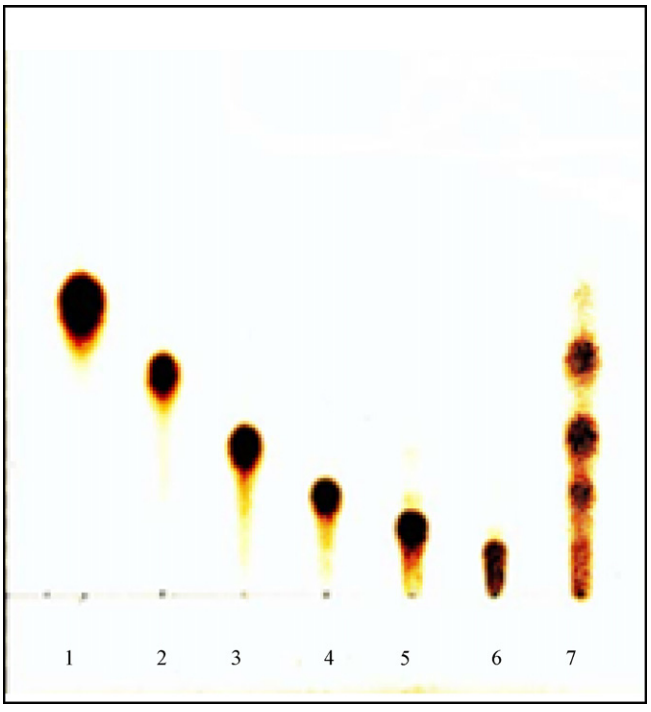

Fig. 2. Thin-layer chromatogram of the hydrolysate of chitosan hydrolyzed by chitosanase in the membrane reactor. Lanes 1–6 are standard chitooligosaccharides: (1) glucosamine, (2) chitobiose, (3) chitotriose, (4) chitotetraose, (5) chitopentaose, and (6) chitohexaose. Lane 7 is the hydrolysate, the mixture of chitooligosaccharides used as substrate in this study.

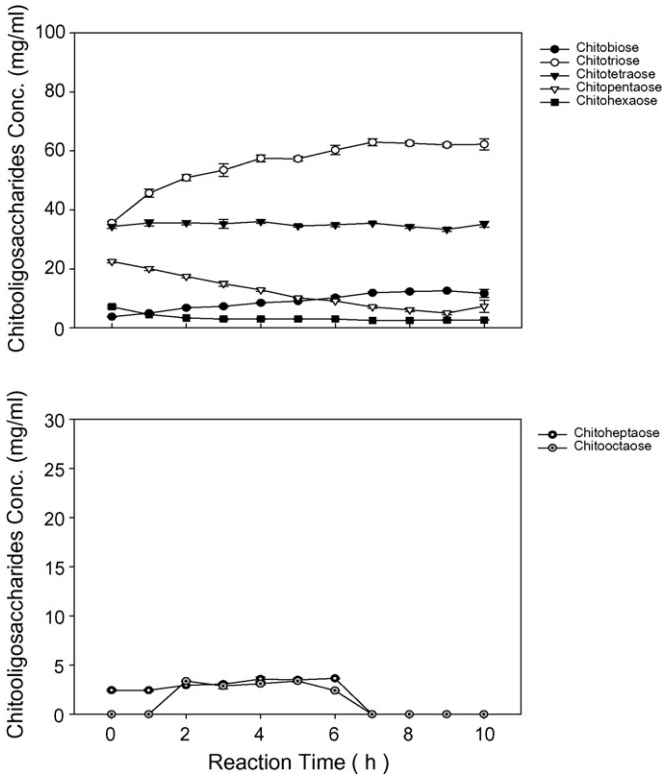

Fig. 3. Changes of chitooligosaccharides composition during enzymatic reaction in bulk aqueous batch reactor at E/S ratio 0.06 unit/mg.

used in this study could not hydrolyze pure dimer and trimer and only slightly hydrolyzed tetramer. It was also found that the chitosanase would hydrolyze pentamer to dimer and trimer, and hydrolyze hexamer mainly to trimer or to dimer and tetramer (Fig. 4).

Besides hydrolysis reactions, the glycosidase enzymes could also catalyze the transfer of a glycosyl moiety from a glycoside derivative onto an acceptor molecule [12,15]. Therefore, pentamer and hexamer not only might be hydrolyzed to dimer and trimer but also could act as the glycosyl acceptors to form higher DP oligomers. Moreover, the accumulation of dimer or trimer increases the opportunity for transglycosylation reaction to occur. Although higher DP oligomers could be formed due to transglycosylation, these chitooligosaccharides also could be easily hydrolyzed in the aqueous system. To enhance the possible transglycosylation reaction or to stabilize the high DP chitooligosaccharides, the low-water content system, reverse micelle, was employed in the subsequent studies.

3.3. Extraction of chitosanase into reverse micelles

To form an effective reversed micellar microreactor, it is essential to solubilize chitosanase in the micelles first. There are three main parameters which would affect the reversed micellar extraction of enzyme, including AOT concentration of the organic phase, NaCl concentration of the aqueous phase, and the temperature. By fixing AOT concentration at 10% while the W_0 was 27.86, the effect of the rest two parameters on the extraction of chitosanase was investigated and the result is

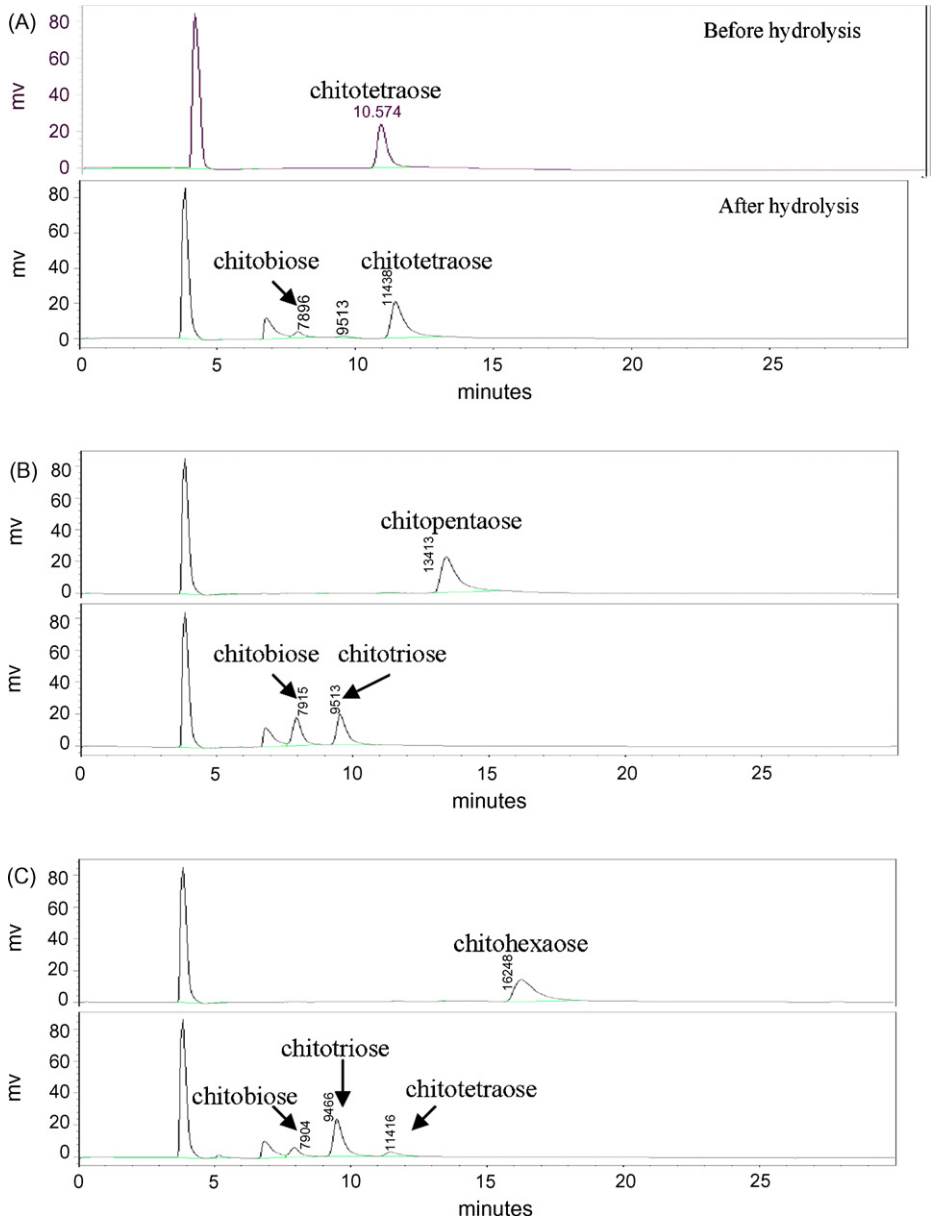

Fig. 4. HPLC profiles of the hydrolysates after enzymatic hydrolysis of pure oligosaccharide standards by chitosanase. (A) Chitotetraose, (B) chitopentaose, and (C) chitohexaose.

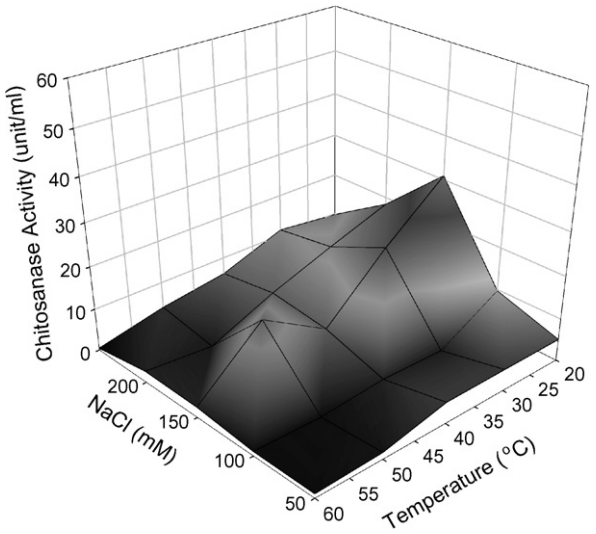

Fig. 5. Effects of temperature and ionic strength on the amount of chitosanase solubilized in the reverse micelles. The reverse micelles were formed in the organic phase containing 10% AOT.

illustrated in Fig. 5. It was found that the highest amount of chitosanase could be solubilized in the micelles at 20 °C and NaCl concentration of 150 mM. On the other hand, when the AOT concentration was 20% and the W_0 was 13.07, the most effective extraction was at 20 °C and 200 mM NaCl concentration (Fig. 6). In addition, it appeared that the organic phase containing 20% AOT extracted more chitosanase than that of 10% AOT. Therefore, the subsequent experiments were carried out at conditions of AOT concentration 20%, 20 °C, and NaCl concentration 200 mM for the formation of reversed micellar microreactors.

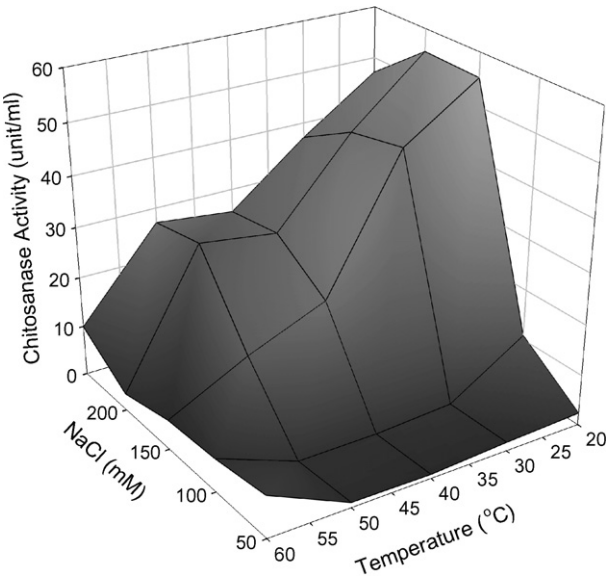

Fig. 6. Effects of temperature and ionic strength on the amount of chitosanase solubilized in the reverse micelles. The reverse micelles were formed in the organic phase containing 20% AOT.

3.4. Effect of water content in reverses micelles (W_0)

Reverse micelles provide not only an effective low-water media but also high concentration of substrate, thus could increase the collision rate of enzyme and substrate in the micelles. In the reverse micelles, water exists as bound water and free water [26]. The bound water interacts with polar head groups of AOT and does not participate in enzymatic reaction. On the other hand, the free water can significantly influence enzymatic activity. A little amount of free water bound to the chitosanase is necessary to maintain its catalytic activity [27]. After the reverse micellar microreactors were formed at 20 °C, the system was moved to a water bath at 40 °C for the enzymatic reaction. The changes of chitooligosaccharides composition during reaction in the reverse micelle at 40 °C, E/S ratio 0.06 unit/mg, and at different W_0 (7.41, 11.86, and 14.82) are shown in Figs. 7–9. When the water content was low (W_0 = 7.41), all chitooligosaccharides decreased and heptamer disappeared after 2.5 h (Fig. 7). Since it has been proven that dimer and trimer could not be hydrolyzed by the enzyme, the phenomena observed could only be attributed to the instability of the reversed micellar system at low-water content. It is known that the size of reverse micelles is dependent on W_0, and the small size reverse micelles with low W_0 sometimes might not be able to accommodate both of the enzyme and substrate [28]. Therefore, the composition of the oligomers in the microreactors might change during processing due to the limitation of the size of the reverse micelles with W_0 = 7.41.

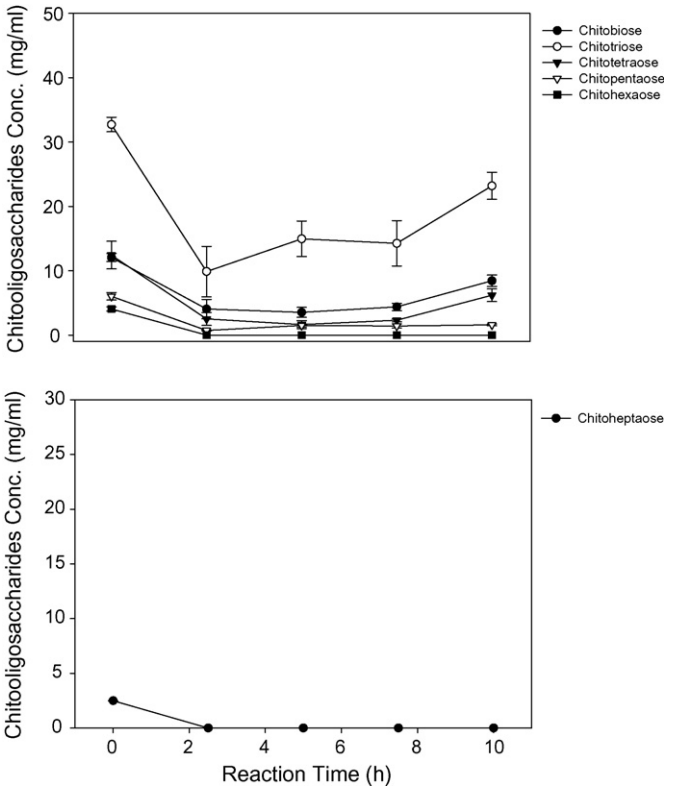

Fig. 7. Changes of chitooligosaccharides composition during enzymatic reaction in the reverse micelle at 40 °C, E/S ratio 0.06 unit/mg, and W_0 7.41.

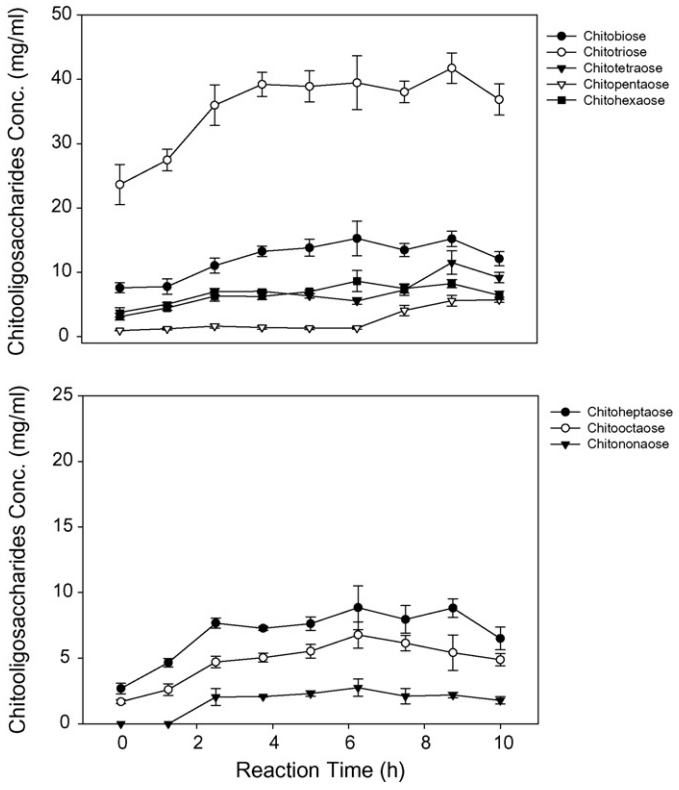

Fig. 8. Changes of chitooligosaccharides composition during enzymatic reaction in the reverse micelle at 40 °C, E/S ratio 0.06 unit/mg, and W_0 11.86.

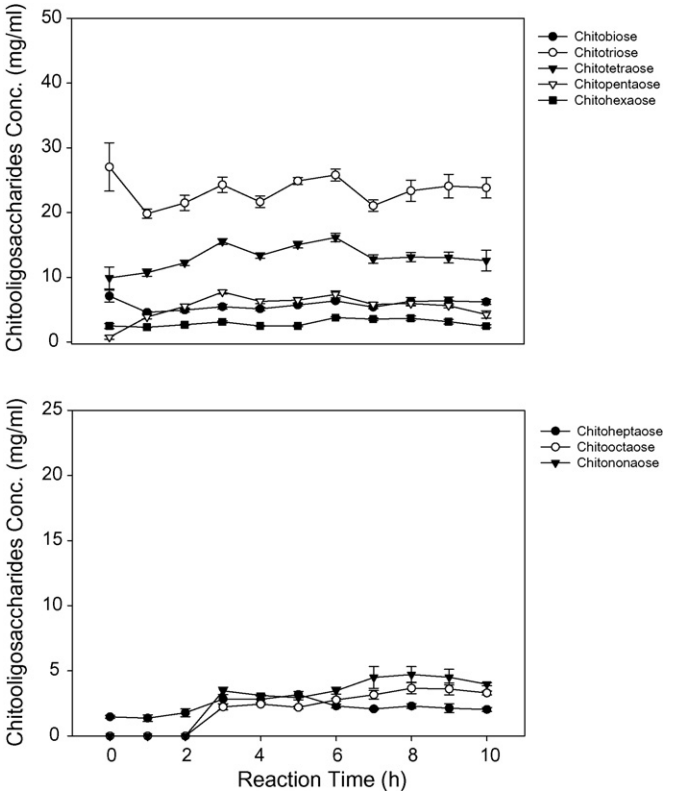

Fig. 9. Changes of chitooligosaccharides composition during enzymatic reaction in the reverse micelle at 40 °C, E/S ratio 0.06 unit/mg, and W_0 14.82.

When the water content of the reverse micelles was relatively higher ($W_0 = 11.86$), trimer increased dramatically and dimer, tetramer, and hexamer increased slightly during the first 4 h of reaction. The concentration of pentamer maintained at a low level throughout the course of the reaction. Heptamer and octamer increased steadily for approximately 6 h after the reaction started, and nonamer appeared after 2.5 h of reaction. In this experiment, transglycosylation might occur (Fig. 8). There was 21.3% pentamer in the original substrate; however, approximately 90% of pentamer was hydrolyzed in the very beginning of the reaction (it should be noted that the data shown at zero time of the process was for the first sample we obtained, when the enzymatic reaction has already proceeded for several minutes), and the concentration of pentamer remained low throughout the reaction. Therefore, pentamer was probably the most sensitive glycosyl acceptor in the transglycosylation reaction in the reverse micelles. The maximum yields of high DP chitooligosaccharides (heptamer ∼ nonamer) were achieved in approximately 6 h. Subsequently, the amount of transglycosylation products, the high DP oligomers decreased whereas tetramer and pentamer increased. The above observations seemed to suggest that the pentamer played an imperative role in not only hydrolysis but also transglycosylation. When the high DP chitooligosaccharides were hydrolyzed after accumulating to a crucial amount, the pentamer was the main product.

For the reactions in the reverse micelles with high-water content ($W_0 = 14.82$), dimer and trimer decreased whereas tetramer and pentamer increased significantly during the initial reaction. After reaction for 3 h, pentamer started to decrease, but octamer and nonamer were produced (Fig. 9). It was also noticed that the amounts of octamer and nonamer formed in the reverse micelles with W_0 of 14.82 were lower than that of 11.86. Theoretically, the high W_0 reversed micellar system is closer to the bulk aqueous system. For the high W_0 reversed micellar system the extra water acted as a reactant to participate in hydrolysis, resulting in a decrease of transglycosylation reaction. Therefore, the chitooligomers in the mixture should be considered as not only the transglycosylation products but also the products of hydrolysis [29].

For a transglycosylation reaction of chitosanase, trimer can be the acceptor and pentamer can be the donor [30]. Our results showed that not only trimer, but pentamer and hexamer also acted as acceptors in the transglycosylation reaction. In addition, pentamer and hexamer served as major donors due to higher hydrolyzing activities of chitosanase toward these two oligomers (Fig. 4). For instance, pentamer might be hydrolyzed to dimer and trimer which could be transferred to another pentamer to form heptamer and octamer. Hexamer could be hydrolyzed to trimer which could be transferred to hexamer to form nonamer.

In conclusion, this study has demonstrated that the reversed micellar microreactor is an effective tool for producing high DP chitooligosaccharides when the proper processing conditions are used, and the mechanism is transglycosylation reaction. It is known that transglycosylation is a kinetically controlled reaction and the product is a potential substrate of glycosidase enzymes. The final reaction yield will be increased by using an

efficient acceptor molecule; increasing donor and acceptor concentration; decreasing water activity [31]. Reverse micelle could provide not only a low-water media but also high concentration of substrate, thus it is an ideal reactor for the transglycosylation reaction to occur. Although nearly all chitosanases are considered inverting enzymes without transglycosylation activity, this research demonstrated that chitosanase could have transglycosylation activity in a reversed micellar microreactor with limited water content. However, it should be emphasized that although transglycosylation was suspected to be the key reaction occurred in the reverse micelles, the mechanism still needs to be elucidated after the chitosanase used in this study being purified and fully characterized.

Acknowledgements

The research was supported by the National Science Council of ROC under the project number NSC 91-2214-E-002-037. The microorganism *B. cereus* NTU-FC-4 was kindly provided by Professor Min-Hsiung Lee of the Department of Agricultural Chemistry, National Taiwan University.

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