PRODUCTION OF CHITOSAN OLIGOSACCHARIDES AT HIGH CONCENTRATION BY CHITOSANASE DIRECTLY IMMOBILIZED ON AN AGAR GEL-COATED MULTIDISK IMPELLER

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Abstract:
An immobilized enzyme bioreactor consisting of an agar gel-coated multidisk impeller was developed for the production of high concentrations of physiologically active pentameric and hexameric chitosan oligosaccharides. Chitosanase was directly immobilized on the agar gel-coated multidisk impeller by a multipoint attachment method. As the hydrolysis proceeded, the concentration of chitosan was increased from the saturation concentration (20 kg/m^3) to 50 kg/m^3 by stepwise addition of chitosan powder while the pH of the reaction mixture was controlled. When the hydrolysis of a 50-kg/m^3 chitosan solution at 50°C was repeated three times with the same immobilized enzyme, the target products were obtained in high concentrations (20 kg/m^3) from each reaction with no reduction in the activity of the immobilized enzyme.

Keywords: chitosan hydrolysis, chitosan oligosaccharides, bioreactor, immobilized enzymes

Introduction

Chitosan oligosaccharides are expected to be utilized as functional foods, medical supplies, and biologically active substances because of their physiological activities. Especially pentameric and hexameric oligosaccharides possess highly beneficial functions such as antimicrobial activities (Uchida et al., 1989), antitumor activities (Tokoro et al., 1988; Suzuki et al., 1986) and immunoenhance effects (Hirano et al., 1991).

Since chitosan oligosaccharides are hydrolyzate of chitosan, therefore, interest in the degradation of chitosan to chitosan oligosaccharides have attracted much attension. Production of the target oligosaccharides at a high concentration is preferable for their application, and it requires a high concentration of the substrate raw material. However, because chitosan solutions are highly viscous even at low concentrations, the use of a high-concentration chitosan solution during hydrolysis would limit the operability of a bioreactor.

To overcome this problem, we have developed a novel bioreactor with an agar gel-coated multidisk impeller bearing directly immobilized chitosanase for the production of high concentration of pentamers and hexamers of chitosan oligosaccharides (Ming et al., 2006). In this paper, we investigated the effective method for operating the bioreactor to obtain a high concentration of the target products. A stepwise addition of substrate chitosan powder was examined in order to increase the substrate concentration. We also studied the stability of the immobilized enzyme in the highly viscous solution by re-using the enzyme for repeated hydrolysis reactions.

Materials and methods

Materials

Chitosanase (EC 3.2.1.132) from *Bacillus pumilus* BN-262 was kindly supplied by Meiji Seika Kaisha (Tokyo, Japan). The final products of chitosan degradation by this endo-type enzyme are dimers and trimers of chitosan oligosaccharides (Fukamizo et al., 1994). Chitosan with 100% of degree of deacetylation was purchased from Funakoshi (Tokyo, Japan). Mean

molecular weight of this chitosan was 370 kDa as determined by a viscometric method (Wang et al, 1991). All other chemicals were analytical or extra-pure grade.

Preparation of the reactor with the agar gel-coated multidisk impeller

The multidisk impeller consisted of five disks of stainless steel wire mesh (18 mesh; diameter, 50 mm) supported by a plastic cross (thickness, 1 mm). The disks were fixed perpendicular to the stainless steel shaft, and a rubber ring (thickness, 1.9 mm) was inserted between each disk to maintain a constant spacing. The impeller was dipped in a 6% agar solution that was kept at 100°C, and then removed from the solution. The agar solution adhering to the mesh was gelatinized into the flat mesh disks at room temperature. The surface of the gel-coated disks became smooth because all the openings in the mesh were filled with the agar gel. The chitosanase was immobilized on the agar-coated disks as described below.

A torque meter (SS-1R, Yamasaki, Kyoto, Japan) was attached to the shaft of the impeller, and a pH probe was inserted into the reactor. The impeller was driven by a variable-speed motor, and the acrylic reactor (diameter, 56 mm; height, 115 mm) was placed in a thermo-statted bath (Fig. 1).

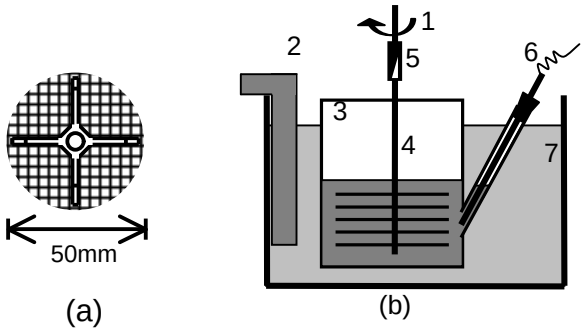

Fig. 1 Experimental apparatus: (a) wire mesh disk and (b) reactor equipped with an agar gel-coated multidisk impeller. (1)Motor, (2) heater, (3) reactor, (4) impeller, (5) torque meter, (6) pH probe, (7) water bath

Preparation of immobilized chitosanase

In this study, chitosanase was immobilized on the agar gel-coated disks by the multipoint attachment method as reported previously (Ichikawa et al., 2002). The gel was activated using glycidol (0.7 mol/L), and then the chitosanase was immobilized on the gel.

Preparation of chitosan solutions

Chitosan solutions with concentrations of 5 and 20 kg/m^3 were prepared as follows. Chitosan powder (1.5 or 6 g) was added to 100 mL of deionized water and dissolved with 70 mL of 1 mol/L lactic acid by stirring. The final pH was adjusted to 5.6 with 5 mol/L NaOH solution, and the volume of the solution was brought to 300 mL to afford either a 5- or 20-kg/m^3 chitosan solution. A 100-mL portion of the solution was used in each hydrolysis experiment.

A 50-kg/m^3 chitosan solution was prepared by adding the chitosan powder directly to the reactor stepwise after the start of the hydrolysis of the 20-kg/m^3 chitosan solution. One gram of chitosan powder was added each time. The timing of addition was determined by monitoring the torque required to agitate the reaction solution. The pH of the starting 20-kg/m^3 chitosan solution was about 3.5 (that is, not adjusted to 5.6). Because the pH of the reaction solution increased with each addition of chitosan powder, 0.2 mL of lactic acid was also added with the powder when the pH of the reaction solution rose above ca. 4. Chitosan

powder was added a total of three times, at which point the final chitosan concentration reached 50 kg/m^3.

Measurement of chitosanase activity

The activity of the immobilized chitosanase was determined according to the method of Uchida and Ohtakara (1988). The concentration of reducing sugar was measured by the modified Schales method (Imoto and Yagishita, 1971) with D-glucosamine (Sigma, St. Louis, MO, USA) as a reference compound. One unit of chitosanase activity was defined as the amount of enzyme that produced 1 μmol of D-glucosamine equivalent in 1 min.

Analysis of chitosan oligosaccharide concentrations

The concentrations of chitosan oligosaccharides, from dimers to hexamers, were determined by high-performance liquid chromatography (HPLC) using a CAPCELL PAK NH_2 column (Shiseido, Tokyo, Japan). The HPLC operating conditions have been described in the literature (Kuroiwa et al., 2002).

Results and Discussion

Effects of impeller speed and enzyme activity at the support surface on production of chitosan oligosaccharides

In a heterogeneous reaction system such as an immobilized enzyme reaction, the intensity of agitation is an important determinant of the reaction rate and mass transfer rate, especially in viscous solutions. Previously, we revealed that the mass transfer rate near the support materials affects the composition of oligosaccharides produced in chitosan hydrolysis using a chitosanase immobilized on an agar gel particle (Kuroiwa et al., 2002). Based on this fact, we studied the effect of impeller speed, which reflected the agitation intensity of the system, on the production of the target oligosaccharides using the bioreactor with the agar gel-coated multidisk impeller. The typical time courses of oligosaccharide concentrations (dimer to hexamer) produced by hydrolysis at two different impeller speeds were shown in Fig. 2-1. In both cases, the concentrations of all the oligosaccharides increased as the reaction progressed at reactions times up to 4–5 h. After the concentrations of pentamers and hexamers (the target intermediate products in this study) reached their maximum values, the concentrations decreased as the reaction proceeded, owing to enzymatic hydrolysis of the intermediates. The total maximum yields of the target products obtained at different impeller speeds were different: a higher impeller speed gave a higher yield. This result shows the promotion of mass transfer near the immobilized enzyme at a high impeller speed is effective to improve the yield of pentameric and hexameric chitosan oligosaccharides.

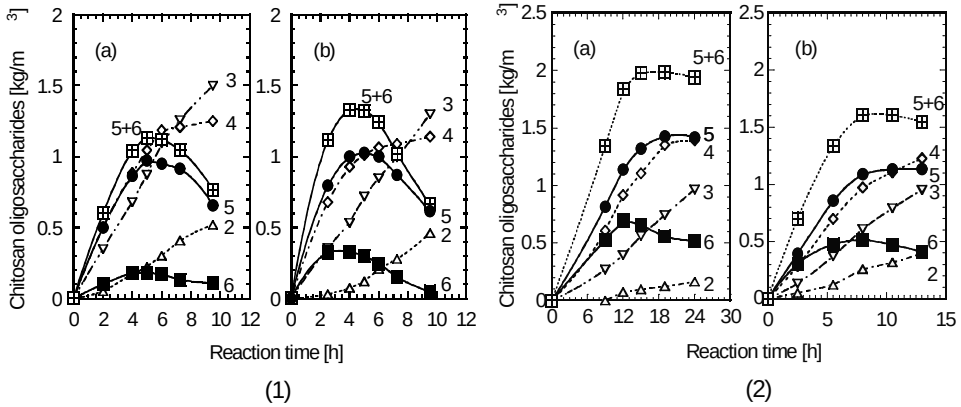

Fig. 2. Time courses of chitosan oligosaccharide concentration produced during hydrolysis of chitosan using the chitosanase immobilized on the agar gel-coated multidisk impeller. (1) Effect of impeller speed: (a) 1 s^{-1}; (b) 2 s^{-1}. (2) Effect of the observed specific activity: (a) 79 U/m^2; (b) 150 U/m^2. Temperature and chitosan concentration were 35 ˚C and 5 kg/m^3, respectively.

In our previous investigation, we also found that the composition of oligosaccharides produced and the maximum yield of the target hexamers and pentamers were affected by the activity of enzyme immobilized on the support surface (Kuroiwa et al., 2002). In this study, the maximum yield of the target products increased with decreasing the observed specific activity of immobilized enzyme per unit surface area at the same impeller speed (Fig. 2-2). The total maximum yield of the target products at 79 U/m^2 (40%) was higher than that at 150 U/m^2 (33%).

Effect of pH on the activity of immobilized chitosanase

In this study, the concentration of chitosan was increased by stepwise addition of chitosan powder as the hydrolysis reaction proceeded. When the chitosan powder dissolved in the reaction solution, the pH of the solution increased. Because the solubility of chitosan decreases with increasing pH, keeping the pH of the reaction solution low is preferable for solubilization of the added chitosan powder. However, the activity of the enzyme also depends on pH. Because both the chitosan solubility and the enzyme activity depend on pH, we set about determining how the pH range varied during the hydrolysis reaction.

To facilitate the dissolution of the chitosan powder, the pH of the reaction solution must be as low as possible in the range over which the hydrolysis reaction can proceed. The optimum pH for hydrolysis of chitosan by immobilized chitosanase on agar gel in lactic acid solution was pH 4–6, whereas the optimum pH for free chitosanase was pH 5.6, determined by our preliminary experiments (data not shown). By means of the following experiment, we confirmed that when the pH was raised again, the activity of the immobilized chitosanase could be recovered even it was exposed to a lower pH environment previously (Fig. 3). The hydrolysis reaction was started at pH 5.6. After 40 min, the pH of the reaction solution was lowered to 3.3 with lactic acid and kept there for 40 min. Then the pH was increased to 4.5 with a 5-mol/L NaOH solution and allowed to remain there for 40 min. During the initial 40-min period, reducing sugar was produced rapidly (1.2×10^{-4} kg/(m^3 s)) because of the high activity of the immobilized enzyme in the optimum pH range. After the pH was lowered to 3.3, the reaction rate became slower (1.9×10^{-5} kg/(m^3 s)) because the pH was not optimum for the activity of immobilized chitosanase. After the pH was increased from 3.3 to 4.5, however, the reaction rate recovered to the same level observed in the initial stage at pH 5.6.

Stepwise addition of chitosan powder for preparing chitosan solution in high concentration

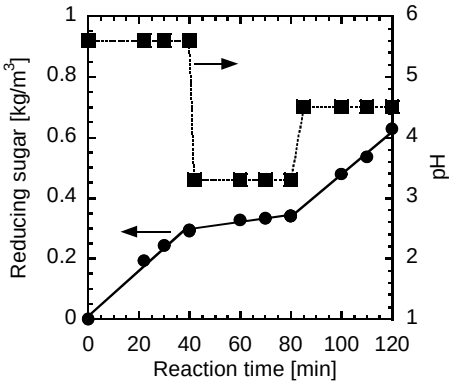

Fig. 3. Activity loss and recovery of immobilized chitosanase by pH change.
Temperature: 35°C; impeller speed: 2 s^{-1}; chitosan solution, 5 kg/m^3. pH: circle; reducing sugar: square.
solution. Observed specific activity: 307 U/m^2; temperature: 35°C; impeller speed: 2 s^{-1}.

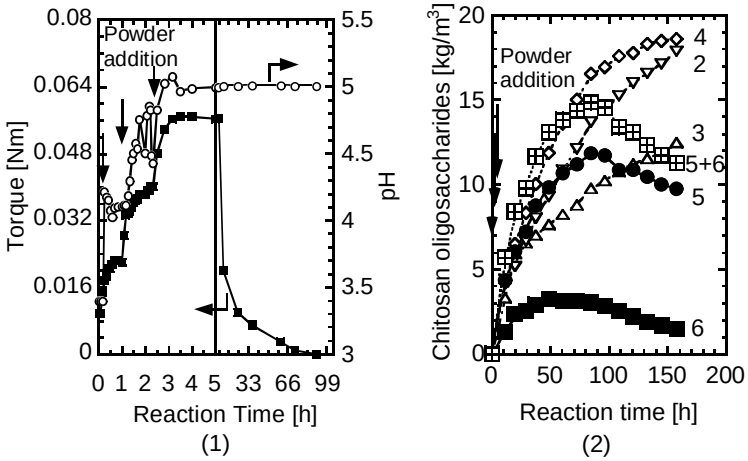

Fig. 4. (1) Changes in pH (circles) and torque (squares) during chitosan hydrolysis by immobilized chitosanase. The chitosan concentration was increased from 20 kg/m^3 to 50 kg/m^3 by stepwise addition of chitosan powder three times. Arrows show the addition of chitosan powder. (2) Time courses of chitosan oligosaccharide concentrations during hydrolysis of this 50-kg/m^3 chitosan solution. Observed specific activity: 307 U/m^2; temperature: 35°C; impeller speed: 4 s^{-1}.

Chitosan solution is highly viscous even at low concentrations of chitosan. For chitosan used in this study, the saturation concentration is about 20 kg/m^3 at pH 5–6 and 35°C in lactic acid solution. Therefore, preparing a solution with a concentration exceeding 20 kg/m^3 in one step is difficult. In this study, stepwise addition of chitosan powder enabled the production of a solution of higher concentration, and the pH of the reaction solution was temporarily lowered to facilitate the dissolution of the added chitosan powder. The timing of powder addition was determined from the change in the torque necessary for agitating the reaction solution, because the torque reflects the viscosity change in the reaction solution: the viscosity increases as the chitosan powder dissolves, and then decreases as the chitosan is hydrolyzed. In this study, as shown in Fig. 4-1, chitosan powder was added consecutively when the torque reached its maximum after the previous addition. At the addition stage, the pH was adjusted to 4-4.5 with lactic acid. When the final chitosan concentration reached 50 kg/m^3, the pH was adjusted to 5.0.

The temporal variation in the concentration of chitosan oligosaccharides produced by the method of stepwise addition is shown in Fig. 4-2. We could obtained a high concentrations of the target pentamers and hexamers at maximum (15 kg/m^3).

Repeated production of chitosan pentamers and hexamers at high concentration

To verify the stability and the re-usability of the bioreactor used in this study, we investigated repeated batch production of chitosan pentamers and hexamers using the same immobilized enzyme. The reaction temperature was set to 50 ˚C to increase solubility of the substrate and to decrease the viscosity of the solution. The substrate concentration was increased to 50 kg/m^3 by the stepwise addition method (Fig. 4). When the concentration of pentamers and hexamers reached a plateau, the impeller and the reactor were washed, and then the next reaction was started. As shown in Fig. 5, the time courses of the degree of hydrolysis and the concentration of the target oligosaccharides were almost identical in the three batch reactions. This result shows that the chitosanase immobilized on the agar gel-coated multidisk impeller was highly stable during the reaction at 50°C in a viscous solution. The maximum concentration of pentamers and hexamers was about 20 kg/m^3 (a 40% yield with respect to the amount of substrate used). To our knowledge, there have been no previous reports of the production of pentamers and hexamers at concentrations higher than 9.5 kg/m^3 using free enzyme (Ming et al., 2006). Thus, the bioreactor presented here has great

advantages in terms of both stability and yield of the target products at high concentrations. The reactor should be useful for the practical production of physiologically active chitosan oligosaccharides. We believe that the production method developed in this study will find application in industry.

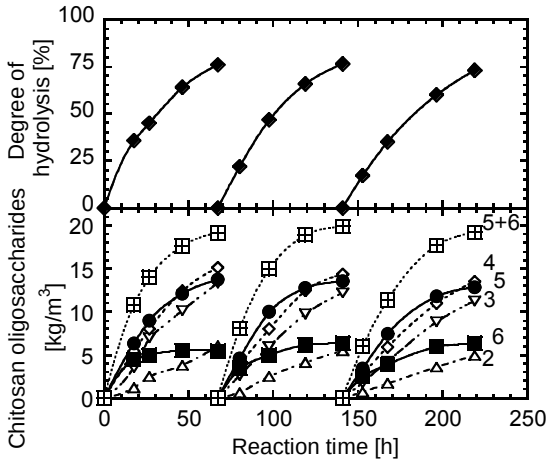

Fig. 5. Repeated hydrolysis of chitosan to produce chitosan pentamers and hexamers at high concentration using the same immobilized chitosanase. Observed specific activity: 209 U/m^2; temperature: 50°C; impeller speed: 4 s^{-1}.

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