Improved production of chitosan oligosaccharides having high physiological activities using immobilized chitosanases

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Abstract:
Continuous production of chitosan oligosaccharides were studied, with regard to the yield of physiologically active pentamers and hexamers of chitosan oligosaccharides. Oligosacchardes were produced by continuous hydrolysis of chitosan using immobilized chitosanases packed into a column-type reactor. The yield of the target products, pentamers and hexamers of chitosan oligosaccharides, was significantly affected by the surface enzyme density on the support material and the mass transfer near the immobilized enzyme. The effects of these factors were summarized as a correlation with Damköhler number (Da), defined as the ratio of the maximum reaction rate to the maximum mass transfer rate near the support surface. Finally, we produced the target oligosaccharides at a high yield (> 35%) stably for a month.
Key words: chitosan oligosaccharides, immobilized enzyme, bioreactor, mass transfer.

Introduction

Chitosan oligosaccharides have attracted much attension because of their physiological activities, such as antimicrobial activity (Uchida et al., 1989), antitumor activity (Tokoro et al., 1988; Suzuki et al., 1986), and immuno-enhancing effects (Hirano et al., 1991). They are expected to be utilized as functional foods and medical supplies. Reportedly, their functional properties depend on their molecular size: higher oligosaccharides such as pentamers and hexamers are especially active. Therefore, products having high content of pentamers and hexamers should be preferred for their effective utilization.

Pentamers and hexamers of chitosan oligosaccharides can be obtained as intermediate products in chitosan hydrolysis. Conventionally, hydrolysis of chitosan has been carried out by a chemical method using a concentrated HCl solusion (Horowits et al., 1957). However, in such method, the yield of higher oligosaccharides is often low due to a difficulty of controlling hydrolysis.

To overcome this problem, we have developed an immobilized chitosanase (Ichikawa et al., 2002; Kuroiwa et al., 2002). Using immobilized chitosanases that can be easily separated from a reaction mixture, we can stop the hydrolysis reaction immediately when the yield of the target intermediate products reaches a maximum. This is a great advantage in use of immobilized chitosanases for producing pentamers and hexamers of chitosan oligosaccharides efficiently.

In this paper, we tried to improve the yield of pentamers and hexamers of chitosan oligosaccharides in continuous hydrolysis of chitosan using a column-type reactor into which the immobilized chitosanases were packed. The effects of the conditions for operating the reactor on the yield of pentamers and hexamers were investigated. We also carried out a long-term production of the target products.

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 (98% deacetylated, mean molecular weight: 58,000) was kindly gifted by Yaegaki Bio-industry (Himeji, Japan). All other chemicals were analytical or extra-pure grade.

Preparation of immobilized chitosanase

Chitosanase was immobilized onto activated agar gel particles by the multipoint attachment method as described previously (Ichikawa et al., 2002). The agar gels were activated with glycidol (0.7 mol/L), and then the enzyme was immobilized on the gel.

Preparation of Chitosan Solution

Chitosan powder (5 or 20 g) was added to deionized water (600 ml) and dissolved with 100 ml of 1-mol/L acetic acid by stirring. The pH was adjusted to 5.6 with 5-mol/L NaOH solution and the solution made up to 1 L affords 5 or 20 kg/m^3 chitosan solution.

Batch hydrolysis of chitosan by free chitosanase

The reaction was carried out in a 200-ml reactor at 35 ˚C. The reaction mixture consisted of free chitosanases and 100 ml of a chitosan solution and was stirred by a magnetic stirrer. The aliquot was taken at regular intervals and heated in boiling water (10 minutes) in order to stop the reaction.

Continuous hydrolysis of chitosan using a packed-bed enzyme reactor

A packed-bed enzyme reactor was used for continuous hydrolysis of chitosan. The immobilized chitosanases were packed into a glass column (135 mm in length, 15.4 mm in diameter) surrounded by the jacket for controlling the column temperature by circulation of water. The continuous hydrolysis of chitosan was achieved by supplying the substrate solution to the reactor continuously. The flow rate of the substrate solution was changed in the range of 0.04 –3.96 ml/min using a constant rate pump. The outlet was taken when the system was considered to be in a steady state.

Analytical methods

The activity of chitosanase was determined according to the method of Uchida and Ohtakara (1988) based on the modified Schales method (Imoto and Yagishita, 1971) with D-glucosamine (Sigma, Co., 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 at 35 ˚C and 5 kg/m^3 of substrate concentration. The degree of hydrolysis of chitosan was determined as a ratio of the reducing sugar concentration of the sample solution to that of the chitosan solution in which chitosan was completely hydrolyzed to dimers and trimers of chitosan oligosaccharides by chitosanase under the above condition.

The concentrations of chitosan oligosaccharides, from dimers to hexamers, were determined by high-performance liquid chromatograghy (HPLC). The HPLC operation was carried out as described previously (Kuroiwa et al., 2002).

Results and Discussion

Batch Hydrolysis of Chitosan by Free Enzymes

The result of batch hydrolysis of chitosan by free enzymes is shown in Fig. 1. Initially, each oligosaccharide concentration increased as the reaction progressed until after approximately 30 minutes. As the reaction proceeded, the concentrations of pentamers and hexamers, the target products in this study, decreased after reaching the maximum values as a result of enzymatic hydrolysis on these intermediates. The maximum concentration of pentamers and hexamers was 1.8 kg/m^3 (36 % (w/w) of the initial chitosan concentration).

On the other hand, the concentrations of dimers and trimers increased throughout the reaction, since they are final products in the hydrolysis reaction. It was observed that no significant change in the maximum yield of pentamers and hexamers occurred by changing the amount of the enzyme, although the reaction time changed when their maximum yield was obtained (data not shown).

From the above results, it was concluded that to produce pentamers and hexamers efficiently the reaction should be stopped when their total concentration reached the maximum value. In order to stop the reaction immediately, therefore, the enzymes should be used in an immobilized form because immobilized enzymes can be separated easily from the reaction mixture.

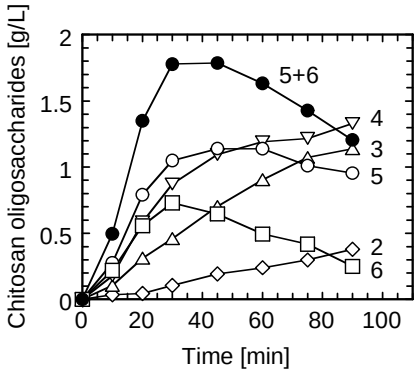

Fig. 1. Typical time courses of chitosan oligosaccharide concentrations in batch hydrolysis of chitosan by free chitosanase. The amount of the enzyme was 63 U in 100 mL of chitosan solution (5 kg/m^3) at 35 ˚C. Number with each symbol represents the degree of polymerization of chitosan oligosaccharides (2 = dimer, 3 = trimer, ...).

Effect of Surface Enzyme Density

The effect of surface enzyme density on the yield of pentamers and hexamers is shown in Figure 2. Since localization of enzymes immobilized on the surface of agar supports was observed by staining of immobilized protein with Coomassie Brilliant Blue, the surface enzyme density was defined as the amount of enzyme immobilized per unit external surface area of support, U/m^2. The external surface area of the agar gel supports was determined from their geometry as they had smooth surfaces. Immobilized enzymes used for experiments had different surface enzyme densities (1.0- 32.6 x 10^3 U/m^2). In all cases, the yield of pentamers and hexamers increased initially as the hydrolysis reaction proceeded, then decreased after reaching the maximum value since further degradation of chitosan occurred. However, the maximum yield of pentamers and hexamers was affected by the surface enzyme density of the immobilized enzymes packed in the reactor. When the surface enzyme density was 1.0 x 10^3 U/m^2, the maximum yield was 37%, as same as the free-enzyme system (36%). As the surface enzyme density increased to 32.6 x 10^3 U/m^2, the yield decreased to 0.18. The results in Fig. 2 indicated that the surface enzyme density should be lower in order to obtain a higer yield of the target products.

Effect of External Mass Transfer

In the case of a solid-liquid heterogeneous reaction system, the mass transfer at the solid-liquid interface is important. In the packed-bed reactor, the external mass transfer rate was changed by the flow rate of the reaction fluid. Different amounts of immobilized chitosanases having the same surface enzyme densities were packed into the reactor, and continuous hydrolysis of chitosan was carried out in the different range of flow rate of substrate solution: a lower amount of immobilized enzymes needs a lower flow rate to obtain the same degree of

hydrolysis. Figure 3 shows relationships between the yield of pentamers and hexamers and the degree of hydrolysis obtained at different flow rate. The yields obtained at higher flow rates were higher than those in lower flow rates at the same period of the reaction. This indicates that the yield of pentamers and hexamers is improved by the increase of the flow rate, that is, an increase of mass transfer rate near the immobilized enzyme at the same period of the reaction. Thus, it is important for effective production of pentamers and hexamers to lower the mass transfer resistance.

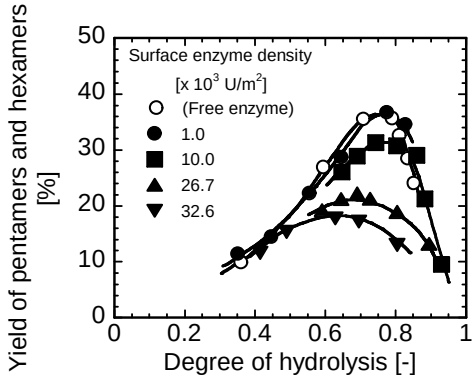

Fig. 2. Effect of surface enzyme density on the yield of pentamers and hexamers based on the initial substrate concentration. Temperature and initial substrate concentration were 35 ˚C and 20 kg/m^3, respectively.

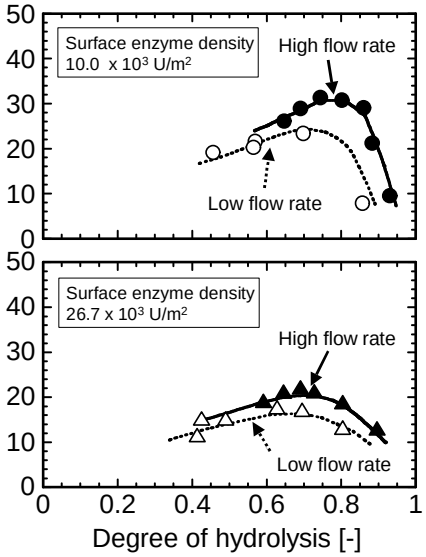

Fig. 3. Effect of external mass transfer on the yield of pentamers and hexamers based on the initial substrate concentration using immobilized chitosanases having different surface enzyme densities. The amounts of immobilized enzymes packed into the reactor were 150 U (open symbols) and 480 U (closed symbols). Other conditions were the same as in Figure 2.

Correlation of the yield of the target products with the Damköhler Number

It has been established that the maximum yield of pentamers and hexamers of chitosan oligosaccharides was affected by the surface enzyme density and external mass transfer. In order to evaluate the effects of these factors comprehensively, we introduced the Damköhler number (Da) defined as follows:

$Da = V_{max} /(k_F C_0)$ = (maximum reaction rate) / (maximum mass transfer rate)

where V_{max} was the maximum reaction rate per unit surface area [kg/(m^2 s)] which was calculated by the rate constant of the free enzyme, the "true" fractional retention of enzyme activity on immobilization (Yamane, 1977), and the amount of enzyme protein loaded on the support surface. k_F was the film mass-transfer coefficient [m/s] estimated using the equation by Wilson and Geankoplis (1966). C_0 was the initial substrate concentration [kg/m^3]. The diffusion coefficient of chitosan [m^2/s] in aqueous solution, which was necessary for estimation of k_F, was evaluated as described previously (Kuroiwa et al., 2002) using a reported equations (Kimura and Nakao, 1985).

The Da values under various conditions were calculated and plotted for the maximum yield of pentamers and hexamers normalized by their maximum yield in the free-enzyme system (Fig. 4). In Fig. 4, the results of batch experiments are also plotted for the Da values calculated in previous study (Kuroiwa et al., 2002). A higher yield was obtained under the condition which gave a smaller Da. The relationships between Da and the maximum yield in batch and continuous experiments were similar in the same range of Da. This indicates that Da is a useful index to obtain a high yield of pentamers and hexamers and an optimum condition for the packed-bed reactor operation can be predicted from the data of preliminary batch experiments.

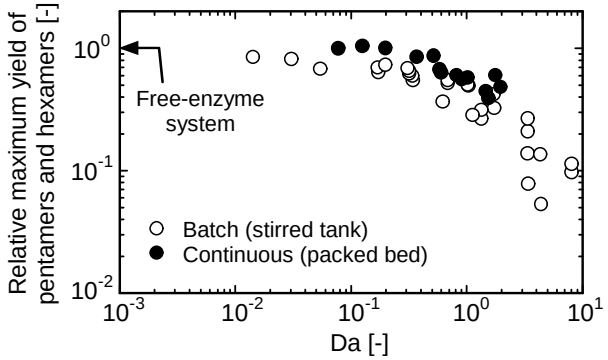

Fig. 4. Correlation of the maximum yield of pentamers and hexamers of chitosan oligosaccharides with Da. The maximum yield is represented as a relative value based on the maximum yield of pentamers and hexamers obtained in the reaction using a free chitosanase (see Fig. 1). Data of the batch reaction are from a previous study (Kuroiwa et al., 2002)

Continuous Production of Physiologically Active Chitosan Oligosaccharides

Since the final objective of enzyme immobilization is to establish a continuous process, continuous production of pentamers and hexamers of chitosan oligosaccharides was carried out using a packed-bed reactor. The time courses of the yield of pentamers and hexamers are shown in Figure 5. Under this operation conditions, Da was calculated as 0.12. In the initial period of continuous operation, pentamers and hexamers of chitosan oligosaccharides were produced in the yield of 38% (7.6 kg/ m^3), which is similar to the maximum yield in a batch system using free enzymes. After about a month, the yield still remained at a high level (35%). It was revealed that the continuous production of pentamers and hexamers of chitosan oligosaccharides could be performed efficiently and stably using the packed-bed enzyme reactor with the immobilized chitosanases.

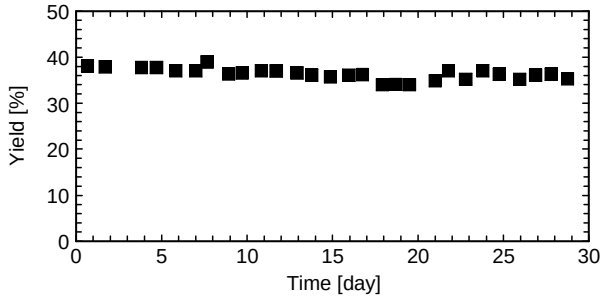

Fig. 5. Continuous production of pentamers and hexamers of chitosan oligosaccharides. The yield is represented based on the initial concentration of chitosan (20 kg/m^3).

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