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# Selective and stable production of physiologically active chitosan oligosaccharides using an enzymatic membrane bioreactor

Takashi Kuroiwa [a,b], Hiroshi Izuta [a], Hiroshi Nabetani [b], Mitsutoshi Nakajima [a,b], Seigo Sato [a], Sukekuni Mukataka [a], Sosaku Ichikawa [a,*]

[a] Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
[b] National Food Research Institute, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

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ABSTRACT

We investigated the production of chitosan oligosaccharides by continuous hydrolysis of chitosan in an enzyme membrane bioreactor, with the goal of improving the yield of physiologically active oligosaccharides (pentamers and hexamers) and achieving operational stability. The bioreactor was a continuous-flow stirred-tank reactor equipped with an ultrafiltration membrane with a molecular weight cut-off of 2000 Da, and the hydrolysis was accomplished with chitosanase from *Bacillus pumilus*. After optimization of the reaction parameters, such as the amount of enzyme, the yield of the target oligosaccharides produced in the membrane bioreactor with free chitosanase reached 52% on the basis of the fed concentration of chitosan. An immobilized chitosanase prepared by the multipoint attachment method was used to improve the operational stability of the membrane bioreactor. Under the optimized conditions, pentameric and hexameric chitosan oligosaccharides were steadily produced at 2.3 g/L (46% yield) for a month. The half-life of the productivity of the reactor was estimated to be 50 d under the conditions examined.



## 1. Introduction

Chitosan oligosaccharides, particularly pentamers and hexamers, are expected to be utilized in medicines and functional foods because of their antibacterial activities, antitumor activities and immunoenhancing effects [1–7]. They are obtained from partial hydrolysis of chitosan. There are two typical methods for producing chitosan oligosaccharides: chemical hydrolysis using acids and enzymatic hydrolysis using chitosanolytic enzymes [8,9]. Generally, because chemical hydrolysis proceeds nonselectively, the product mixture obtained by means of the chemical method contains large amounts of D-glucosamine, the monomer of chitosan [10]. In contrast, chitosanolytic enzymes having appropriate selectivity produce little D-glucosamine [1,11,12]. Therefore, enzymatic hydrolysis in a bioreactor has the potential to be effective for the production of chitosan oligosaccharides, especially higher oligosaccharides such as pentamers and hexamers.

The development of membrane technology has led to a new type of bioreactor in which continuous reaction occurs simultaneously with separation of the product from the reaction mixture [13]. A membrane bioreactor contains an enzyme and a membrane with the appropriate pore size and physicochemical properties. The substrate is continuously fed into the reaction mixture while the products are continuously withdrawn in the permeate. The size of the product molecules in the permeate can be controlled by proper selection of the pore size of the membrane. Therefore membrane bioreactors can be expected to be effective for the production of desirable chitosan oligosaccharides by chitosan hydrolysis. Jeon and Kim [14] reported a dual reactor system that consists of a packed-bed bioreactor and a membrane bioreactor for enzymatic production of chitosan oligosaccharides. By using ultrafiltration (UF) membranes with different pore sizes they successfully controlled the molecular weight distribution of the oligosaccharides obtained, although they did not report quantitative values of the yields of physiologically active oligosaccharides including pentamers and hexamers.

In this study, we investigated enzymatic hydrolysis of chitosan in a membrane bioreactor for the purpose of efficiently producing pentameric and hexameric chitosan oligosaccharides. The reactor operation conditions were optimized with regard to the total yield of pentameric and hexameric chitosan oligosaccharides, because product mixtures containing large amounts of these compounds

* Corresponding author. Tel.: +81 29 853 4627; fax: +81 29 853 4605.
*E-mail address:* sosakui@sakura.cc.tsukuba.ac.jp (S. Ichikawa).

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are desirable for practical applications. In addition, long-term operation of the reactor was studied. Even though membrane bioreactors are usually operated in continuous reaction mode, to our knowledge, continuous production of chitosan oligosaccharides for longer than 48 h [14] using membrane bioreactors has not been reported. This is probably due to the low stability of chitosanases in the membrane bioreactor: generally enzymes are used in free (soluble) form and free chitosanases are not stable enough at their reactive temperature (35–50 °C). Therefore, we examined the utilization of an immobilized chitosanase in the membrane bioreactor to improve enzyme stability, with the goal of developing a bioreactor for the selective and stable production of useful chitosan oligosaccharides.

## 2. Materials and methods

### 2.1. Materials

Chitosanase from *Bacillus pumilus* BN-262 was obtained from Meiji Seika Kaisha (Tokyo, Japan). This endo-type enzyme can hydrolyze both glucosamine–glucosamine and *N*-acetylglucosamine (non-reducing-end side)–glucosamine (reducing-end side) linkages in chitosan molecules, and the final products of chitosan degradation by this enzyme are dimers and trimers of chitosan oligosaccharides [12]. The enzyme contained 13.2% protein, as determined by the Bradford method [15] using bovine serum albumin as the standard. The enzyme was used without further purification.

Chitosan (Chitosan 10B, 100% deacetylated) was purchased from Funakoshi Co. (Tokyo, Japan). Its average molecular weight was 370,000 Da, as determined by the viscometric method [16].

All other chemicals were analytical or extra-pure grade and were obtained from commercial sources.

### 2.2. Batch hydrolysis of chitosan by free chitosanase

Chitosan was dissolved in 0.1 M acetic acid to a concentration 5 g/L. The solution was adjusted to pH 5.6 with 5 M NaOH. The enzyme concentration in the reaction mixture was 480 U/L. The hydrolysis reaction was carried out at 35 °C with stirring by a magnetic stirrer. Aliquots were removed at regular intervals and heated in boiling water for 10 min to stop the reaction.

### 2.3. Batch hydrolysis of chitosan by immobilized chitosanase

Chitosanase was immobilized on the surface of 1-mm cubic agar gels by the multipoint attachment method [17–19] as described previously [20]. Support gels were activated with 0.7 M glycidol. No enzyme detachment from the support was observed during three repeats of the batch experiment in a previous study [20].

Ten grams (wet weight) of the support gel with the immobilized chitosanase was added to 100 mL of the chitosan solution (5 g/L, pH 5.6), and the mixture was gently stirred by a turbine agitator. The temperature of the reaction mixture was kept at 35 °C. Aliquots were removed at regular intervals and filtered immediately to remove the immobilized enzyme to stop the reaction.

### 2.4. Continuous hydrolysis in a membrane reactor

Fig. 1 is a schematic diagram of the continuous-flow stirred-tank membrane reactor system used in this study. The system consisted of the membrane separation equipment (Model UHP-62K, Advantec Co., Tokyo) and the reservoir for the substrate solution. The effective membrane area was $2.7 \times 10^{-3}$ m$^2$. The membrane was a polysulfone flat-sheet UF membrane with a molecular weight cut-off of 2000 Da (GR-90PP, Dow Danmarks A/S, Denmark). Initially, 100 mL of chitosan solution (5 g/L, pH 5.6; prepared as described in Section 2.2) and chitosanase in free or immobilized form were added to the reactor. Then the same chitosan solution was fed into the membrane reactor from a reservoir by $N_2$ gas pressure. The volumetric feed rate of the chitosan solution corresponded to the permeation flux multiplied by the effective membrane area; therefore, the volume of the reaction mixture inside the reactor was kept at a constant value. Throughout operation, the reaction mixture was stirred by a magnetic stirrer, and the temperature of the reactor was kept at 35 °C.

### 2.5. Analysis

The activity of chitosanase was determined from the increase in the amount of reducing sugars produced by hydrolysis of chitosan [21]. The concentration of reducing sugars was measured by the modified Schales method [22] with D-glucosamine as a reference compound. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of D-glucosamine equivalent in 1 min at a substrate concentration of 5 g/L and 35 °C.

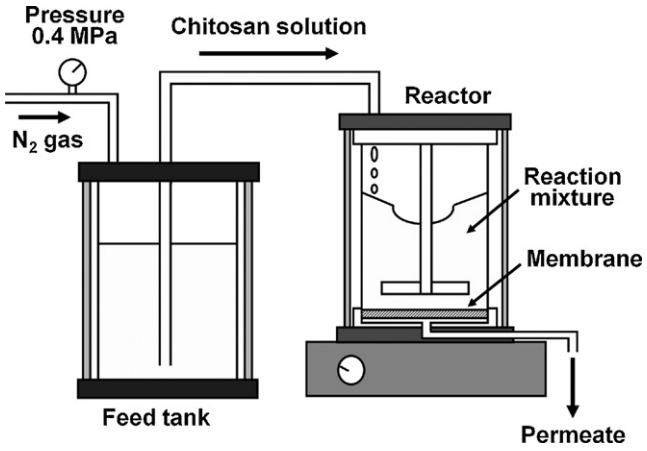


**Fig. 1.** Schematic diagram of the continuous-flow stirred-tank membrane reactor.

The concentrations of dimeric to hexameric chitosan oligosaccharides were measured by high-performance liquid chromatography (HPLC) using a CAPCELL PAK $NH_2$ column ($\phi$ 4.6 mm × 250 mm) from Shiseido Co. (Tokyo, Japan). The HPLC operation was carried out with a mixture of acetonitrile and water (75/25, v/v) as the mobile phase at a flow rate of 1 mL/min. The column temperature was kept at 45 °C. The concentration of each oligosaccharide was calibrated with a chitosan oligosaccharide mixture (Seikagaku Corporation, Tokyo, Japan) as the standard.

## 3. Results and discussion

### 3.1. Batch hydrolysis of chitosan using free chitosanase

We measured the time course of chitosan oligosaccharide concentrations during the batch hydrolysis of chitosan using free chitosanase (Fig. 2). The concentrations of the target pentamers and hexamers initially increased with time and then decreased after peaking. These oligosaccharides were intermediate products of the hydrolysis reaction and were degraded by further enzymatic reaction. The maximum total concentration of pentamers and hexamers was 2.8 g/L, corresponding to 56% of the initial chitosan concentration. In contrast, the concentrations of dimers and trimers, which were the final products of the hydrolysis reaction, increased throughout the reaction. From these results, we concluded that efficient production of the target products required that the progress of the hydrolysis reaction should be appropriately controlled.

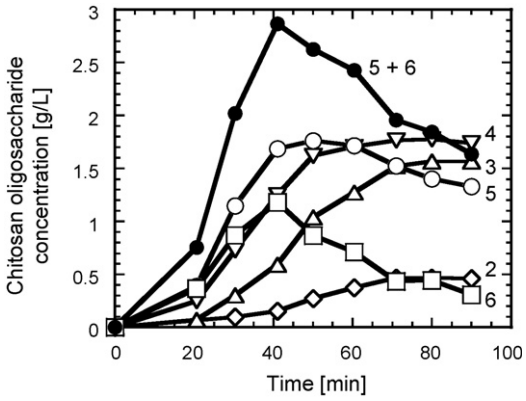


**Fig. 2.** Time course of chitosan oligosaccharide concentrations during chitosan hydrolysis using free chitosanase. Numbers next to the symbols correspond to the degree of polymerization of the oligosaccharides. Enzyme concentration, 480 U/L; substrate concentration, 5 g/L; reaction temperature, 35 °C.