Purification of Physiologically Active Chitosan Oligosaccharides by Means of Nanofiltration Membrane

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We used nanofiltration membranes to study the purification of physiologically active chitosan oligosaccharides (pentamers and hexamers). Calculations based on the experimental data of batch filtration suggested that the content of the target oligosaccharides in the product mixture could be improved from 30% to 77% by continuous diafiltration under optimized conditions.

Key words : chitosan oligosaccharide / nanofiltration membrane / rejection factor / diafiltration

1. Introduction

Chitin (a polymer of N-acetyl-\(\alpha\)-glucosamine) and chitosan (a polymer of \(\alpha\)-glucosamine) make up a large proportion of the earth’s biomass. Chitosan is the only natural source of cationic polysaccharides, and chitosan and its derivatives have been widely utilized for preparing functional materials with unique properties. Recently chitosan oligosaccharides have attracted much attention for their use in functional foods and medical supplies because they exhibit many beneficial physiological activities, such as antibacterial, antitumor, and immuno-enhancing activities 1~6). Reportedly, these activities strongly depend on molecular weight; pentamers and hexamers are especially active, so product mixtures with a high content of pentamers and hexamers are preferred.

Previously, we investigated the enzymatic production of chitosan oligosaccharides, paying particular attention to the yield of pentamers and hexamers 7~13). Using bioreactors with immobilized chitosanases prepared by the multipoint attachment method 7), we produced chitosan oligosaccharide mixtures with high yields of pentamers and hexamers (40 ~ 50% relative to the amount of chitosan used) 9~13). However, during partial hydrolysis of chitosan, the production of lower oligosaccharides, such as dimers and trimers, is unavoidable because the site of chitosan hydrolysis is random. If the lower oligosaccharides could be eliminated from the final product mixture, the resulting mixture would be enriched in the higher oligosaccharides and thus exhibit stronger physiological activities.

Chitosan oligosaccharides are usually separated by column chromatography 1, 14), and an appropriately designed chromatography method can provide pure individual oligosaccharides. However, chromatography often generates a large amount of chemical waste, such as concentrated acids, and the oligosaccharides are recovered in dilute solution. Concentration of the products requires evaporation, freeze-drying, or both, and these processes consume a lot of energy. Here, we evaluated a new process for the purification of chitosan oligosaccharides using nanofiltration (NF) membranes. Membrane technology is commonly used in the modern food industry and has been used for the purification and concentration of various oligosaccharides 15~18). Membrane filtration is a pressure-driven...
method capable of purifying and concentrating a sample solution without the need for heat, toxic reagents, or energy consumption. Heat is particularly problematic for chitosan oligosaccharides because they undergo the Maillard reaction (reaction between a primary amino group and a carbonyl group) to produce brown substances during heat treatment[19]. Therefore, we believe that membrane filtration is the most promising method for purifying and concentrating chitosan oligosaccharides.

First, we found appropriate NF membrane and filtration conditions by means of batch filtration experiments. Second, we evaluated the purification performance in a continuous diafiltration operation by means of a simulation based on the filtration properties obtained experimentally.

2. Materials and methods

2.1 Materials

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[20].

Chitosanase from Bacillus pumilus BN–262 was obtained from Meiji Seika Kaisha (Tokyo, Japan). The enzyme was immobilized on activated agar gels, as reported previously[7], and used for the hydrolysis of chitosan.

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

2.2 Preparation of chitosan oligosaccharide solution

Chitosan oligosaccharide solution was prepared in a membrane bioreactor with the immobilized chitosanase, as reported previously[13]. The oligosaccharide composition of the solution was determined by high–performance liquid chromatography (HPLC) and found to be as follows: dimer, 0.4 g/L; trimer, 1.4 g/L; tetramer, 1.7 g/L; pentamer, 1.2 g/L; hexamer, 0.3 g/L; total oligosaccharides, 5.0 g/L.

2.3 Purification of chitosan oligosaccharides by NF

The chitosan oligosaccharide mixture was subjected to batch NF experiments in a membrane test module (batch–type lab–test unit, C40–B, Nitto Denko, Co. [Osaka, Japan]; effective membrane area, 32 cm²). We used NF membranes (G5, G10, and G20) manufactured by Desalination Systems (CA, USA) from a polyamide composite; the membranes had nominal salt rejection values of 65%, 30%, and 10%, respectively. A schematic of the NF apparatus is shown in Fig. 1. The chitosan oligosaccharide solution (0.2 L) was placed in the test module, which was pressurized (1 ~ 5 MPa) with N₂ gas. The solution was stirred with a magnetic stirrer at an agitation speed of 8.3 s⁻¹, and the temperature was kept at 35 °C during the filtration operation. Permeate flux was determined from the weight of the permeate per unit time. The filtration operation was stopped when the total volume of the permeate solution reached 0.05 L, at which time both the permeate and the retentate were recovered and then analyzed for oligosaccharide concentration. The observed rejection factor, \( R_{\text{obs}} \), was calculated with the following equation which was obtained from mass–balance consideration in batch filtration:

\[
R_{\text{obs,n}} = \log \left( \frac{C_{r,n}}{C_{0,n}} \right) / \log \left( \frac{V_0}{V} \right) \tag{1}
\]

where \( C_r \) [g/L] is the oligosaccharide concentration in the retentate; \( C_0 \) [g/L] is the oligosaccharide concentration in the solution before the filtration experiment; \( V_0 \) and \( V \) [L] are the volumes of the retentate before and after the experiment, respectively; and \( n \) is the degree of polymerization of the chitosan oligosaccharide.
2.4 Determination of chitosan oligosaccharide concentration

The concentrations of chitosan dimers to hexamers were determined by HPLC with a CAPCELL PAK NH2 column (Shiseido Co., Tokyo, Japan) under conditions described previously13).

3. Results and discussion

We first examined the permeation characteristics of the three types of NF membrane (G5, G10, and G20) for water and the solution of chitosan oligosaccharides produced in an immobilized enzyme bioreactor13). Fig. 2 shows the permeate fluxes of water and the oligosaccharide solution under various applied pressures for each NF membrane. For all the membranes, the permeate flux increased linearly with increasing applied pressure. The permeate flux decreased as the nominal salt rejection of the membrane increased; this result reflected the fineness of the membrane structure. For all the membranes, the permeate flux of the oligosaccharide solution was lower than that of deionized water under identical conditions. The lower fluxes of the oligosaccharide solutions compared with those of pure water would be the result of osmotic pressure from the accumulation of oligosaccharides on the membrane surface, clogging the membrane pores by the solutes, or both.

Fig. 3 shows the relationship between applied pressure and the observed rejection factor of each oligosaccharide for the G5 (a), G10 (b), and G20 (c) membranes. Numbers next to the symbols correspond to the degree of polymerization of the oligosaccharides. The stirrer speed was 8.3 s⁻¹ and the temperature was 35 °C.
factors of the oligosaccharides followed this trend with the G5 membrane, and all oligosaccharides were rejected at 5 MPa, indicating that the oligosaccharides in the mixture could be concentrated under these conditions without any loss of the oligosaccharides. In contrast, with the G10 and G20 membranes, decreases in the observed rejection factors of the higher oligosaccharides were observed as the pressure was increased (Fig. 3b and c). We attributed this result to the concentration polarization phenomenon. If concentration polarization occurs near the membrane surface, the observed rejection factor often decreases with increasing applied pressure: an increase in applied pressure promotes the formation of a concentrated layer of solutes near the membrane surface owing to the relatively high permeation flux of the solvent, and thus the actual concentration of solute at the membrane surface becomes higher than that of the bulk solution in the retentate phase. When the decrease in the bulk phase concentration of solute exceeds the increase in actual rejection at the membrane surface, the observed rejection factor calculated with Eq. 1 decreases with increasing applied pressure. Similar results were obtained for other oligosaccharides.

To determine the optimal conditions for efficient separation of pentamers and hexamers from the lower oligosaccharides, we had to find conditions under which the rejection of pentamers and hexamers was high and rejection of dimers, trimers, and tetramers was low: that is, conditions under which the ratio of the observed rejection factors of dimers, trimers, and tetramers to those of pentamers and hexamers \( R_{obs,2+3+4}/R_{obs,5+6} \) was small. The experimental values

![Figure 4](image_url)

**Fig. 4** Effect of applied pressure on rejection selectivity of chitosan oligosaccharides for the G5 (circles), G10 (squares), and G20 (triangles) membranes. The stirrer speed was 8.3 s\(^{-1}\) and the temperature was 35℃.

![Figure 5](image_url)

**Fig. 5** Calculated changes in the chitosan oligosaccharide composition for continuous diafiltration using the G5 membrane at 1 MPa: (a) initial composition, (b, c, and d) composition in the retentate during continuous diafiltration calculated from Eq. 2 with dilution rates of 3.0 (b), 7.0 (c), and 10.0 (d). The values of \( R_{obs} \) for dimers (0.30), trimers (0.35), tetramers (0.76), pentamers (0.81) and hexamers (1.0) in Fig. 3a were used for the calculation.
of $R_{\text{obs},2+3+4}/R_{\text{obs},5+6}$ are plotted against applied pressure in Fig. 4. The smallest $R_{\text{obs},2+3+4}/R_{\text{obs},5+6}$ value was obtained with the G5 membrane at 1 MPa. Under these conditions, the observed rejection factors of the pentamers and hexamers were 0.8 and 1.0, respectively.

The calculated oligosaccharide composition for a diafiltration operation under the above conditions is shown in Fig. 5. The oligosaccharide composition was calculated with Eq. 2 for the experimentally obtained $R_{\text{obs}}$ values with the G5 membrane at 1 MPa (Fig. 3a):

$$C_{\text{r,n}} = C_{0,n} \exp \{ - (V_p/V_r) (1 - R_{\text{obs},n}) \} \quad (2)$$

where $C_r$ [g/L] is the oligosaccharide concentration in the retentate, $C_0$ [g/L] is the oligosaccharide concentration in the solution before the filtration experiment, $V_p$ [L] is the accumulated volume of the permeate which is equal to the amount of wash water, $V_r$ [L] is the volume of the retentate which is constant, and $n$ is the degree of polymerization of the chitosan oligosaccharide. The dilution rate, $D$ [-], is defined as $D = V_p/V_r$.

As shown in Fig. 5, the content of hexameric oligosaccharides increased with increasing dilution rate because of their high rejection factor ($R_{\text{obs}} = 1.0$). In contrast, the content of dimers and trimers decreased with increasing dilution rate. At a dilution rate of 10, the retentate contained only tetramers, pentamers, and hexamers, and the total content of the target pentamers and hexamers reached 77% of the total amount of chitosan oligosaccharides. This result suggests that the content of physiologically active oligosaccharides in the final product mixture could be increased by means of the NF membrane technique. We believe that our combination of a membrane bioreactor for selective hydrolysis of chitosan and the NF technique described in this report will be useful for producing high-value oligosaccharide products from chitosan. In addition, the lower oligosaccharides that are removed from the oligosaccharide mixture can be reused, for example, as substrates for the lipase-catalyzed modification of chitosan oligosaccharides for producing functional surfactants.

In conclusion, we purified physiologically active chitosan pentamers and hexamers by using NF membranes. Using a G5 membrane (nominal salt rejection, 65%) at 1 MPa of applied pressure, we could purify the useful oligosaccharides efficiently from the starting chitosan oligosaccharide mixture. Calculated results for a diafiltration operation suggest that the total content of pentamers and hexamers could be increased to 77% in the final product under appropriate conditions. We expect this purification process to be useful for the production of high-value oligosaccharide products.

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