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Purification of DP 6 to 8 chitooligosaccharides by nanofiltration from the prepared chitooligosaccharides syrup

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Abstract

Background: Chitooligosaccharides (COS) with degrees of polymerization (DP) 6 to 8 are degraded from chitosan, which possess excellent bioactivities. However, technologies that could purify them from hydrolysis mixtures in the narrow DP range (984 to 1,306 Da) are absent. The objective of this research is to purify DP 6 to 8 COS by nanofiltration on the basis of appropriate adjustments of the feed condition.

Methods: Syrup containing DP 6 to 8 COS at different concentrations (19.0 to 46.7 g/L) was prepared. A commercial membrane (QY-5-NF-1812) negatively charged was applied. Experiments were carried out in full recycle mode, so that the observed COS retentions were investigated at various transmembrane pressures (6.0 to 20.0 bar), temperatures (10°C to 50°C), and pHs (5.0 to 9.0). Then, the feasibility of separation of DP 6 to 8 COS was further studied by concentration ratio under optimum conditions.

Results: The results indicate that the purification of DP 6 to 8 COS by nanofiltration NF is feasible. It was found that the permeate flux was 95.0 L/(m^2 h) at 10.0 bar, while it reached to 140.0 L/(m^2 h) at 20.0 bar, and it increased with feed temperature, but the membrane pores were also swelled by heating and led to an irreversible wastage of target oligomers. Additionally, the retention behaviors of chitooligosaccharides are significantly influenced by pH.

Conclusions: Although glucosamine and dimer were permeatable at low pH, their retention ratios were remarkably varied from 0.458 to 0.864 when pH was 9.0. With the interaction of hydrogen bonds, structural curling and overlapping of chitooligosaccharides were formed. Consequently, the rejection of chitooligosaccharides at various pHs is variable. Spray-dried products were finally characterized by the matrix-assisted laser desorption/ionization time-of-flight mass spectrum. The spectrum identified the distributions of hexamer, heptamer, and octamer. Combined with high-performance liquid chromatography profiles, the purity and yield of DP 6 to 8 chitooligosaccharides were up to 82.2% and 73.9%, respectively.

Keywords: Nanofiltration; Chitooligosaccharides; Degree of polymerization; Separation; MALDI-TOF-MS

Background

Chitooligosaccharides (COS) are defined as the partially degraded products of chitosan or low-molecular-weight chitosan (LMWC) with a degree of polymerization (DP) ranges from 2 to 20 [1]. As shown in Figure 1, their molecular structures are linear oligosaccharides composed of 2-amino-2-deoxy-D-glucopyranose and 2-acetamino-2-deoxy-D-glucopyranose (Figure 1(A)) units which are linked by β(1 → 4) glycosidic bonds [2].

According to many previous reports, COS possess a series of attractive bioactive properties, including antibacterial [3], anticoagulant [4], antimicrobial [5], antioxidant [6], anticancer [7], hypolipidemic [8], and immune-stimulating [9] effects. Based on these excellent advantages mentioned before, COS are responsible for practical applications in beverage processing [10], functional ingredients [11], and biomedicines [12], which are different from chitosan and

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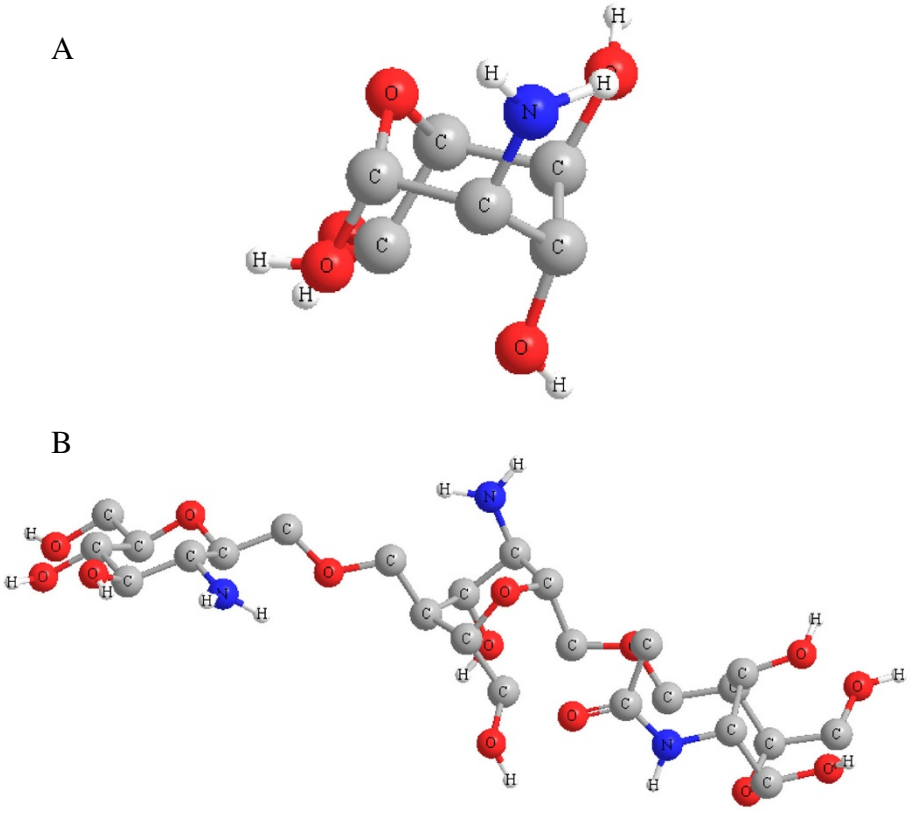

Figure 1 Molecular structures of glucosamine and DP 3 chitooligosaccharide. Structurally, chitooligosaccharides, including glucosamine and trimer, are linear oligosaccharides composed of 2-amino-2-deoxy-D-glucopyranose and 2-acetamino-2-deoxy-D-glucopyranose units which are linked by β(1 → 4) glycosidic bonds. **(A)** The molecular structure of glucosamine. **(B)** The molecular structure of DP 3 chitooligosaccharide.

chitin except for their contributions on food packaging [13]. Nowadays, hybrid enzymatic hydrolysis has become the ideal technology for COS preparation due to its high efficiency and little structural modification [14]. Unfortunately, the products after enzymatic degradation were just intermediate ones [15]. Suitable methods should be used to separate target COS from mixtures coexisting in the solution, such as the high molecular weight of chitosan, proteins (enzymes), and inorganic salts.

Conventionally, COS is purified by various chromatographies. Fan et al. succeeded in obtaining COS by macroporous resins from fermentation broth, and the productivity of target products could go up to 90% (*w*/*w*) under optimum conditions [16]. Meanwhile, Cabrera and Custem reported that the concentrations of COS could be analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrum (MALDI-TOF-MS) [17]. Although the chromatography technology is feasible to remove irrelevant components and refine COS in terms of DP, it is only accepted in laboratory analysis but far from scale-ups. In practice, several negative problems inevitably appear during continuous using of chromatography, including the demands on cleaning and regeneration of column packing, adsorption capacities of COS, and relatively high production cost [18,19].

Recently, there is a growing attention on nanofiltration (NF) applications, especially for COS preparation. Kim et al. reported that several instruments were used to prepare COS, the details of which could be summarized in immobilized enzymatic columns and ultrafiltration (UF) membrane reactors [2]. Han et al. demonstrated the desalination feasibility of NF-40 membrane for chitobiose solution. Under acidic conditions, the interception of solutes ranks as chitobiose > glucosamine > Na^+ > H^+ [20]. Furthermore, the steric and electrostatic effects are inferred according to the sequence. Han et al. also studied the influence of three membranes (DL, DK, NTR-7540) on COS separation and drew identical conclusions [21]. In a word, NF has been proved to be an effective technology for the separation and purification of COS.

Chitosan was originally hydrolyzed by enzymes, which resulted in the coexistence of various DP COS, glucosamines, and salts. It is evident that there is a great difference in molecular weight among the solutes. Also, the wider ranges of hydrolysis products make it difficult to improve the purity and yield of COS. For instance, DP 6

to 8 COS (984 to 1,306 Da) plays an important role in cancer curing [22]. Therefore, this study is to investigate the separation performance of DP 6 to 8 COS at different solution properties and present a promising purification technology by NF.

Methods

Materials

The raw syrup containing DP 6 to 8 COS was made from chitosan by enzymatic hydrolysis. Chitosan was supplied by Yunzhou Biochemistry Co., Ltd. (Shandong, China). The enzyme mixture (chitosanase from *Streptomyces griseus* - EC 3.2.1.132; cellulase from *Trichoderma* - EC 3.2.1.4) was obtained from Golden-Shell Biochemical Co. (Zhejiang, China). DP 2 to 8 COS and glucosamine standards were purchased from Huicheng Biochemical Co., Ltd. (Shanghai, China). All chemicals used in the NF operation and high-performance liquid chromatography (HPLC) analysis were analytical grade or chromatographic grade, respectively. Deionized water (conductivity <3.0 μs/cm) for membrane cleaning was produced by ion exchange.

Preparation of chitooligosaccharide syrup

Chitosan (91.5% degree of deacetylation) was dissolved in acetic acid (1%, *w*/*v*) with stirring, and pH was adjusted to 5.3, and then kept at 45°C. The chitosan concentration was 5% (*w*/*v*). Combined enzymes (75 U/g) based on chitosan were added into the chitosan solution and hydrolyzed for 6 h, and then, the hydrolysis was terminated by immersion in a boiling water bath. Finally, a UF membrane module (QY-3-UF-1812, AMFOR Inc., Newport Beach, US) was applied to remove enzymes at 50°C. After cooling to ambient temperature, the syrup was diluted to various concentrations for NF separation.

Membrane

Purification experiments were operated in a pilot setup (Figure 2). The setup composes of a feed tank, a high-flux pump, a membrane vessel, and a flowmeter. Moreover, the inlet and outlet pressures were metered by two pressure gauges, and the feed temperature could be regulated by a circulating cooling water system surrounding the feed tank.

The membrane module employed was an organic polymer composite with spiral-wound structures (QY-5-NF-1812, AMFOR Inc., Newport Beach, US). The membrane module is measured as 1.8 in. (4.6 cm) in section diameter and 12 in. (30.5 cm) in length, which is so-called 1812 type module. Also, it is measured by an approximate molecular weight cut-off (MWCO) of 500 Da, which is close to the molecular weight of DP 4 COS. As described in Table 1, the membrane is negatively charged that can tolerate a maximum pressure up to 25.0 bar. The effective surface area is 0.2 m^2. The temperature and pH tolerance range cover from 0°C to 60°C and 4 to 12, respectively.

Membrane permeate flux

In this study, the permeate flux is represented as the average one in process. The average permeate flux (J_v) is calculated by Equation 1, as follows:

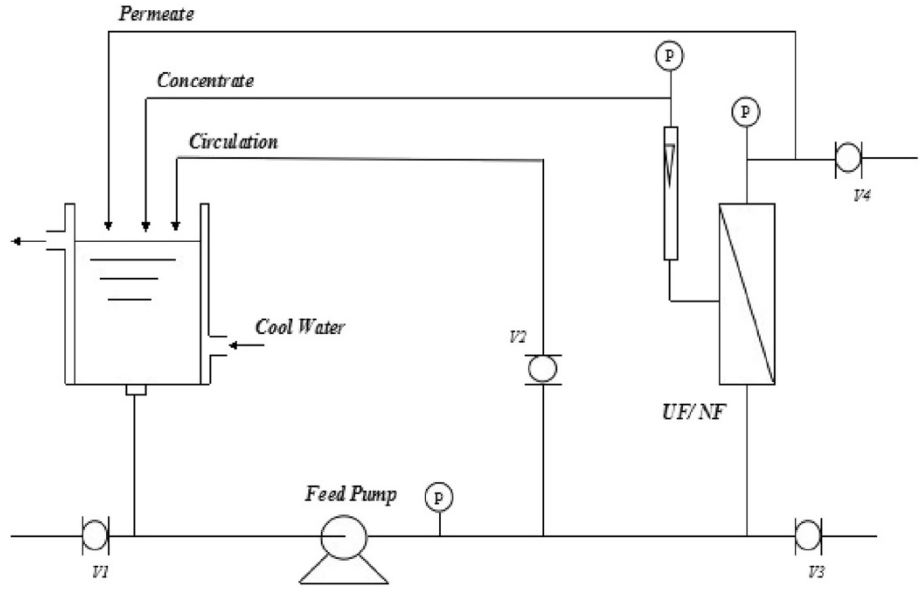

Figure 2 Brief scheme of the nanofiltration system. Purification experiments were operated in batch equipment for laboratory use. V1 to V4 were represented as valves, and P are pressure gages.

Table 1 Information of NF membrane module

Membrane index	Parameter
Type	1812
Texture	Organic polymers
Filtration area (A_m)	0.2 m^2
MWCO	500 Da
Temperature	0°C to 60°C
pH	4 to 12
Pressure	<25.0 bar

$$J_v = \frac{V_p}{A_m \times t} \quad (1)$$

where J_v is the average permeate flux of the membrane [L/(m^2 h)]; V_p is the volume of permeate accumulated in testing time (L); A_m is the effective areas of membrane (m^2); t is the testing time (h).

Observed retention ratio

Retention ratio is also one of the most important factors in membrane separation. The observed retention ratio is described by Equation 2:

$$R_{obs} = 1 - \frac{C_p}{C_b} \quad (2)$$

where R_{obs} is the observed retention ratio of solute (%); C_p is the concentration of solute in the permeate (mol/L); C_b is the concentration of solute in the feed (mol/L).

Full recycle mode of NF

Initially, 5.0 L raw syrup was added, and the recycling flow rate was adjusted to 360 L/h, which represented the optimum rotation condition for the working pump. The effects of transmembrane pressure (TMP), operation temperature, and pH on NF performance were successively carried out. All the permeate were flowed back to the feed tank, while the J_v and retention behaviors of DP 6 to 8 COS at different concentrations were measured after the renewed conditions were stable for 15 min. It is worth being noted that two of the parameters mentioned must be constant when the third is variable. During the process, the temperature was controlled at 50°C ± 2°C by a heat exchanger surrounding the feed tank, except for the experiments to investigate the effects of temperature on the NF retention on DP 6 to 8 COS.

Purification of DP 6 to 8 chitooligosaccharides

Purification experiments for DP 6 to 8 COS were executed in batch mode. Under the optimized conditions obtained from the preliminary experiments, the concentrate stream was circulated back to the feed tank, whereas the permeate was collected individually. Considering the practical capacity of the feed tank, 7.0 L of diluted syrup (C_b = 19.0 g/L) was added firstly. Every 1.0 L of extra diluted syrup should be supplied as soon as the permeate was equally removed. Certainly, the systematic temperature during NF was maintained by cooling water. After adding all the syrup (16.0 L), the process was terminated until the volume of permeate reached 14.0 L (2.0 L syrup left in the tank). The effect of the concentration ratio on the purity of DP 6 to 8 COS was confirmed by flux and rejections.

HPLC analysis

The concentrations of glucosamine and DP 2 to 8 COS were analyzed by an HPLC system (Shimadzu 10A, Shimadzu, Kyoto, Japan) equipped with a high-performance sugar column (Shodex Asahipak NH2P-50 4E, Shodex, Kyoto, Japan) and an RI detector. The mobile phase consists of methyl cyanide and pure water with the ratio of 70:30 (v/v). The column temperature was maintained at 30°C, and the flow velocity was kept at 1.0 mL/min. All the solutes were measured in the form of single-arranged peaks. In general, the glucosamine was firstly eluted, and then, the dimer and trimer were sequentially characterized due to the adsorption strength difference to the stationary phase. The distributions of COS were quantified by integrating peak areas.

MALDI-TOF-MS analysis

MALDI-TOF-MS analysis of COS was carried out using Shimadzu AXIMA Performance matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Shimadzu, Kyoto, Japan). All spectra were measured in the reflector mode by external calibration. The laser was scanned at a scale from 500 to 1,500 Da. An aqueous

Table 2 The component analysis of raw materials for the NF process

Chitosan (%)	Total Proteins[a] (mg/L)	Glucosamine[a] (g/L)	DP 6 to 8 chitooligosaccharides[a] (g/L)
1.0	1.4 ± 0.3	0.4 ± 0.1	9.6 ± 1.2
2.0	2.7 ± 0.2	0.9 ± 0.1	19.0 ± 1.5
3.0	3.3 ± 0.4	1.4 ± 0.2	28.3 ± 1.7
4.0	2.9 ± 0.3	1.8 ± 0.2	37.6 ± 2.4
5.0	1.4 ± 0.3	2.3 ± 0.4	46.7 ± 2.1

[a]The data were determined by three parallel measurements.

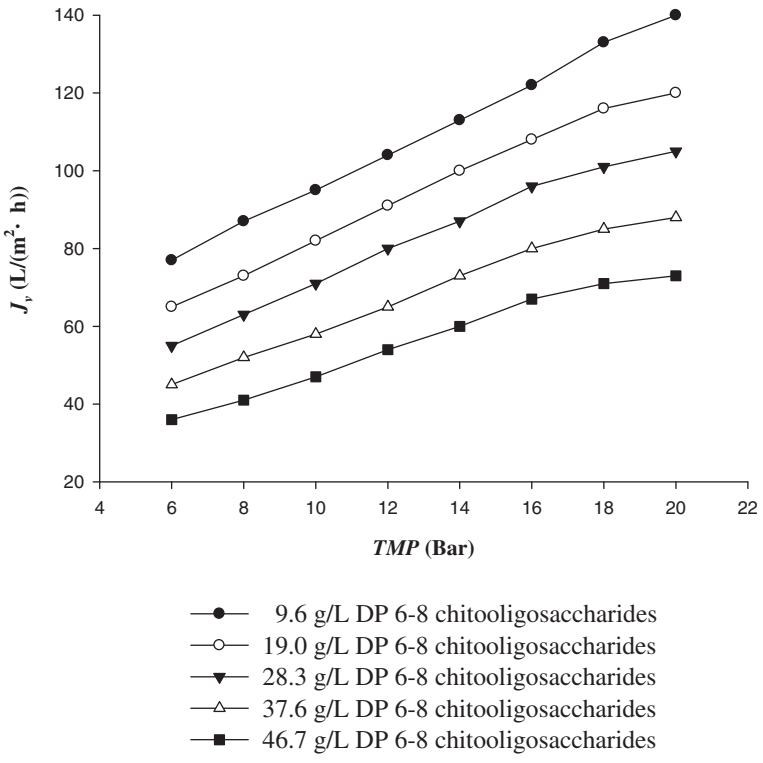

Figure 3 TMP vs. J_v of chitooligosaccharide syrup at 50°C during NF separation. J_v of chitooligosaccharide syrup with different $C_{chitosan}$ ranged by transmembrane pressure (6.0 to 20.0 bar). The permeate fluxes increased with the pressure, whereas they decreased with the increasing concentration of DP 6 to 8 chitooligosaccharides.

solution of 2,5-dihydroxybenzoic acid (DHB, 100 mg/mL) was used as the matrix.

Statistical methods

All parameters and experimental results were obtained with means ± SD of parallel tests. The data was analyzed by Statistix 9.0 software (Analytical Software, Tallahassee, FL, USA).

Results and discussion

Characterization of chitooligosaccharide syrup

As the raw material for NF process, the COS syrup was defined as the permeate of UF that was pretreated after enzymatic hydrolysis. Summarized by the previous tests, 10.0 L of chitosan hydrolysates were prepared and purified by UF, while a 2.0-L concentrate and 8.0-L permeate were separately bulked at each concentration level due to the cross-flow filtration mode.

Table 2 shows that the principle components of COS syrup varied with the changeable concentrations of chitosan (1.0% to 5.0%, *w*/*w*) after UF. It demonstrated that most of the target products permeated through the UF membrane, while the blocking layer adhered to by macromolecular solutes was slightly affected. However, there was still a little amount of proteins ($C_{protein}$ = 1.4 to 2.9 mg/L) that remained, which was partially caused by the temperature effect and concentration polarization. Besides the protein content in the purified syrup, its calculation data was in good agreement with the R_{obs} during the UF process, which was scaled from 0.996 to 0.998 (the data was not shown). The phenomenon suggested that the tested UF membrane was sensitive to the protein rejections.

In addition, with the increment of crude concentration ($C_{chitosan}$), the contents of glucosamine (C_s) and DP 6 to 8 COS ($C_{DP\ 6\ to\ 8}$) were also dramatically increased. For example, C_g and $C_{DP\ 6\ to\ 8}$ at $C_{chitosan}$ = 2.0% were 0.9 and 19.0 g/L, respectively, while those at $C_{chitosan}$ = 5.0% changed to 2.3 and 46.7 g/L, respectively. The tendency indicates that the preparation and separation process of DP 6 to 8 COS was rarely affected by the syrup concentration. According to the data listed in Table 2, the conclusion could be drawn that the purification of hydrolysates by the UF membrane was highly performed. What is more important, the excellent elimination of unexpected impurities may greatly benefit for the next NF treatments.

Effects of transmembrane pressure on permeate flux

Figure 3 illustrates that the permeate fluxes (J_v) of COS syrup at different $C_{chitosan}$ increase with the TMP from 6.0 to 20.0 bar. Moreover, it shows a linear relationship

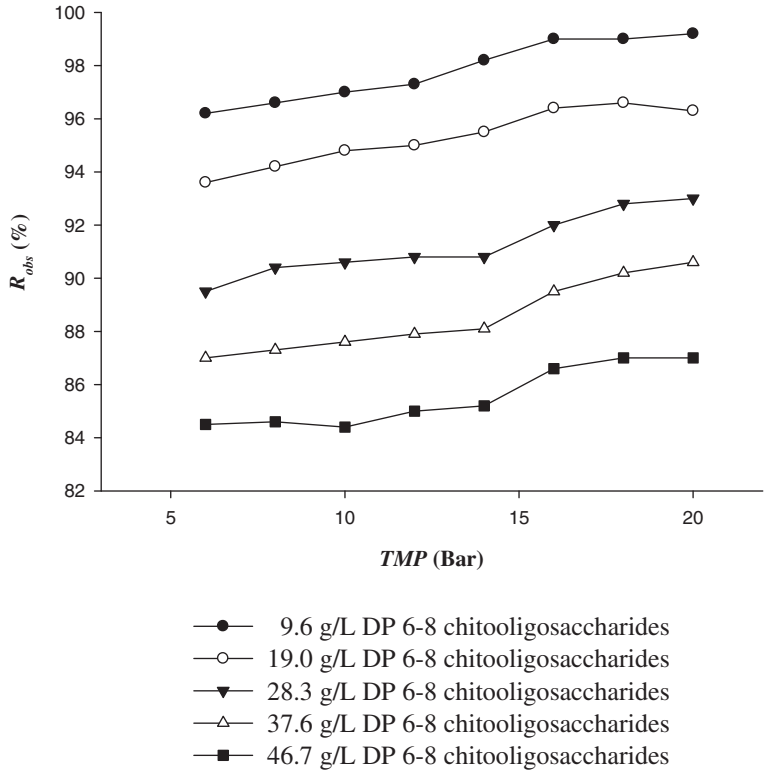

Figure 4 TMP vs. R_{obs} of DP 6 to 8 chitooligosaccharides at 50°C during NF separation. It pictured the retentions of DP 6 to 8 chitooligosaccharides when the TMP was set between 6.0 and 20.0 bar. The R_{obs} of DP 6 to 8 chitooligosaccharides were significantly judged by viscosity and pressure applied.

between TMP and J_v. For instance, the J_v was 95.0 L/(m^2 h) at 10.0 bar for the 9.6-g/L COS solution, whereas it elevated to 122.0 and 140.0 L/(m^2 h) at 16.0 and 20.0 bar at the same syrup concentration, respectively. Membrane compaction reduces the mass transfer resistance and enhances the velocity on membrane surface, and thus, leads to an increasing permeate volume of feed solution in regular intervals.

Conversely, J_v decreased with the increment of concentration of DP 6 to 8 COS. It could be well explained by the interaction of TMP and the accumulation effects. On the one hand, the solutes would be greatly accumulated by the driving pressure, which is beneficial for the formation of cake layer around the boundary of the membrane. As a result, the membrane pore would be partially blocked and result in a decrement of J_v. On the other hand, the concentration polarization was also exacerbated with the increase of the sugar concentration. When the osmotic pressure was 16.0 bar, J_v declined from 108.0 to 67 L/(m^2 h), although the concentration of filtrated syrup ascended from 19.0 to 46.7 g/L.

Effects of TMP on R_{obs} of DP 6 to 8 COS

As shown in Figure 4, the retentions of DP 6 to 8 COS varied with the TMP, and the R_{obs} of target oligosaccharides were significantly affected by the density and pressure applied. The influence of feed concentration on separation behaviors was emphasized at 28.3 g/L. Due to the existence of DP 6 to 8 COS, the increased concentrations were inevitably brought to a higher permeation of solutes. However, it was also found that the retention coefficient of DP 6 to 8 COS was immobile at higher-pressure areas (18.0 to 20.0 bar). The details could be explained by the steric hindrance interaction between solutes and membrane pores.

Additionally, the R_{obs} of DP 6 to 8 COS increased with TMP, which indicate that the concentration polarization was a subordinate factor for the separation conditions in this case. As an alkaline molecule, the rejection properties of COS are significantly affected by the Donnan effects [23]. When the concentration was kept at 28.3 g/L, the R_{obs} of solute decreased with the decrease of the pressure (6.0 to 16.0 bar). According to the Donnan theory, dielectric exclusion impels the increasing sugar accumulation, which is responsible for the considerable R_{obs} of DP 6 to 8 COS. Therefore, it could be seen that the dropping of observed retention at a high-pressure range (18.0 to 20.0 bar) was similar. The corresponding results were of the same order with reported phenomena by Zhang [24]. Impressive retention proportions of DP 6 to 8 COS at all selected concentrations and applied pressures were performed.

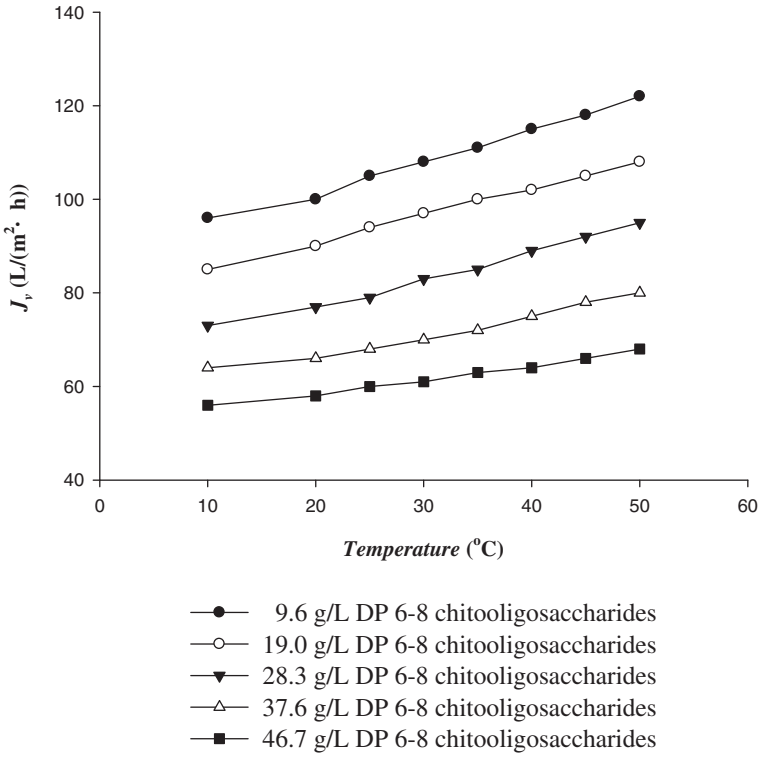

Figure 5 Temperatures vs. J_v of chitooligosaccharide syrup at 16.0 bar in NF process. It depicted the effects of J_v on operation temperature, as well as the concentration variations of DP 6 to 8 chitooligosaccharides. Membrane pores were stretched by thermal expansion at high temperature, which reduced the rate of concentration polarization and improved the membrane flux.

Effects of operation temperature on permeate flux

Figure 5 illustrates the experimental data of membrane fluxes over operation temperature, as well as the concentration variations of DP 6 to 8 COS. It hypothesized that the syrup concentration was a constant and that the permeate flux during full recycle mode in the NF separation process was proportional to the temperature, which resulted from membrane swelling and the decrease of syrup viscosity under elevated temperature. As shown in Figure 5, the permeate flux was 79.0 L/(m^2 h) at 25°C when the concentration was set at 28.3 g/L. After the temperature rose to 40°C, the permeate flux was correspondingly reached to 89.0 L/(m^2 h).

To be detailed, the membrane pores were stretched by structural modification when thermal expansion was interposed by external temperature. At the same moment, the increment of temperature meant for a decline of solution viscosity but a rise both in Reynolds number (*Re*) and mass transfer coefficient (*k*). Followed by the rules analyzed above, all solutes in the process preferred to move to the bulk part of the syrups, which reduced the rate of concentration polarization and thus improved the membrane flux.

However, excessive temperature was negative to preserve the stability of DP 6 to 8 COS. Because of the special structures that the amino group locates on C-2 sites in every monomer unit linked by β (1 → 4) glycosidic bonds, the velocity of the Maillard reaction was motivated by high temperature. Certainly, further studies are needed to understand the mechanism of the Maillard reaction and its derivative products in permeate during the filtration process.

Effects of operation temperature on R_{obs} of DP 6 to 8 COS

The retention manners of DP 6 to 8 chitooligosaccharides in different concentrations of syrup are shown in Figure 5, as the temperature varied from 10°C to 50°C. As expected, the retentions of DP 6 to 8 chitooligosaccharides decreased with the increment of temperature. All curves coincide with the conclusion drawn from Figure 5. That is, the thermo swelling of membrane structures promoted more DP 6 to 8 chitooligosaccharides permeated through the NF membrane from the concentrate side. Specifically, the R_{obs} of DP 6 to 8 chitooligosaccharides was 0.912 in 20°C, whereas it diminished to 0.895 at 50°C with the concentration of 37.6 g/L. The transformation was apparently introduced that the R_{obs} of solutes decreased with a boosting temperature that forced the fluid properties of

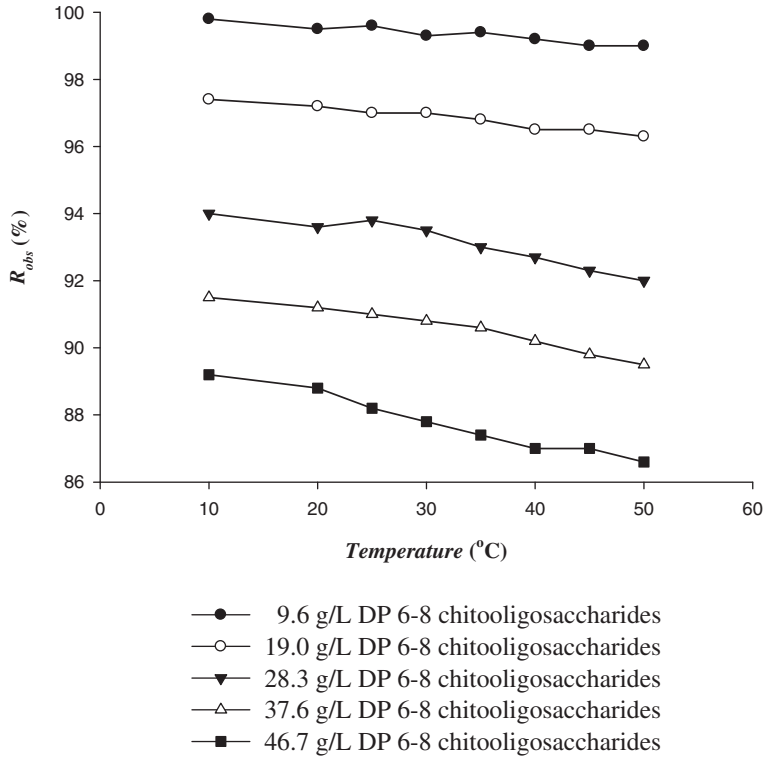

Figure 6 Temperatures vs. R_{obs} of DP 6 to 8 chitooligosaccharides at 16.0 bar in NF process. It reflected the retention behaviors of DP 6 to 8 chitooligosaccharides in different concentrations when the temperature varied from 10°C to 50°C. The retentions of DP 6 to 8 chitooligosaccharides decreased with the temperature increased.

chitooligosaccharide syrup to be a Newtonian liquid and hence resulted in a decrease in viscosity.

Besides, it is also worth noting that the charge effect shifted to become a major factor in the retention behaviors of DP 6 to 8 COS at variable concentrations (Figure 6). Under the same condition (i.e., TMP and temperature), the R_{obs} of hexamer, heptamer, and octamer summed to 0.994 at a concentration of 9.6 g/L, but it declined to 0.874 at a concentration of 46.7 g/L. That could be explained by the Donnan steric pore model (DSPM) theory assumed by Bowen et al. [25]. Given that the negative charges were evenly distributed inside the selected membrane (QY-5-NF-1812), all of the opposite-charged ions as H^+ and NH_4^+ in the solution would be attracted thus the transport of COS was facilitated. Overall, the J_v and retention trends were both restricted by the concentration of syrup and its operation temperature.

Effects of pH on R_{obs} of COS

The effects of pH ranged from 5.0 to 9.0 on rejections of glucosamine and DP 2 to 8 COS were investigated. Singles situated in the area of 13.0 to 19.0 min represent COS within DP 6 to 8. As shown in Figure 7, the R_{obs} of COS increased with pH, especially for the monomer, dimer, trimer, and tetramer. In the chromatogram of NF concentrate at pH 5.0, it was found that nearly all of COS (DP ≤4) had permeated through the membrane, whereas higher DP were effectively rejected. On the contrary, when the syrup was adjusted to a basic condition (pH = 9.0), the R_{obs} of the monosaccharide and disaccharides arrived at 0.458 and 0.864, respectively, even that the trimer and tetramer completely escaped from the permeate. The reason for this phenomenon might be due to the inter- and intramolecular hydrogen bonds, which further resulted in the unequal curling and overlap in structures. Similar results on R_{obs} and inference for COS have been taken by Han et al. [20]. The stability of the primary structure was broken by hydrogen bonds and restabilized spontaneously. During the recombination process, the curling extent would extremely impact the viscosity and stereospecific blockage of molecules. The structural explosion guided the poor affinity of solutes with the membrane and led to a high resistance of permeation. Furthermore, the rejections of DP 6 to 8 COS, formulated from the concentrate and permeate profiles, were scarcely impacted via pH options (Figure 7). The results indicated that the applied driving force was centered on oligomers of DP ≤4 under acidic conditions and sequentially spread to higher polymers as the pH increased. DP 6 to 8 COS is expected to be completely rejected by the NF membrane at a wider

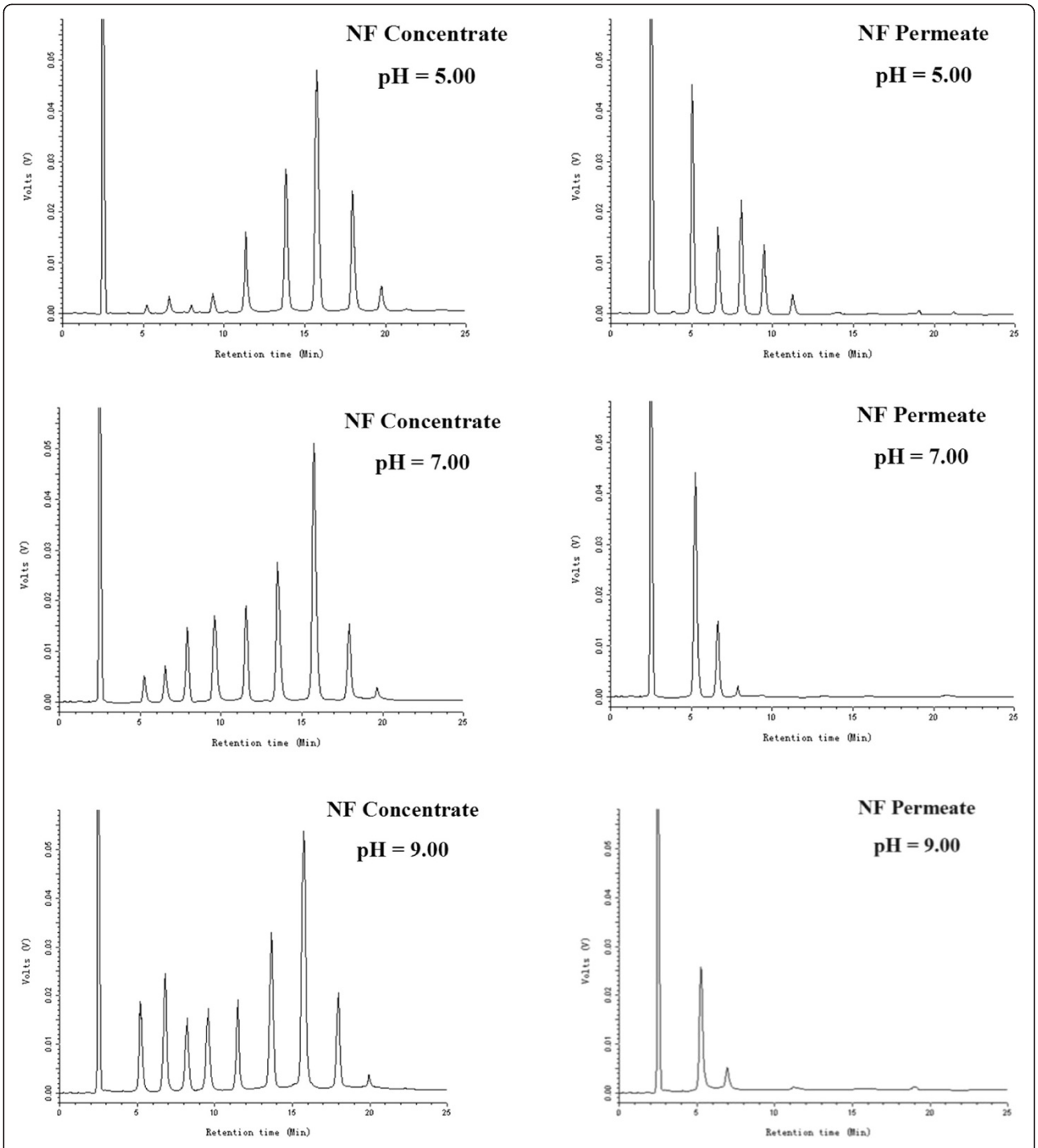

Figure 7 pH values vs. R_{obs} of chitooligosaccharides at 16.0 bar and 40°C in NF process. The effects of pH ranged from 5.0 to 9.0 on rejections of glucosamine and DP 2 to 8 chitooligosaccharides were investigated. The R_{obs} of chitooligosaccharides increased with pH, especially for the monomer, dimer, trimer, and tetramer. In addition, the flexibility of chitooligosaccharides was well conducted by pH.

pH range (5.0 to 9.0). More important, the flexibility of COS was well conducted by the adjustment of pH. Nevertheless, in view of the objective of purification, a lower pH should be adopted for the impurity elimination. Interestingly, several small-sized peaks that were followed by octasaccharides were also characterized at 19.5 min (Figure 7). Due to the randomness of enzymatic hydrolysis on chitosan, this was probably attributed to the nonamers (DP 9 COS) existing in the syrup.

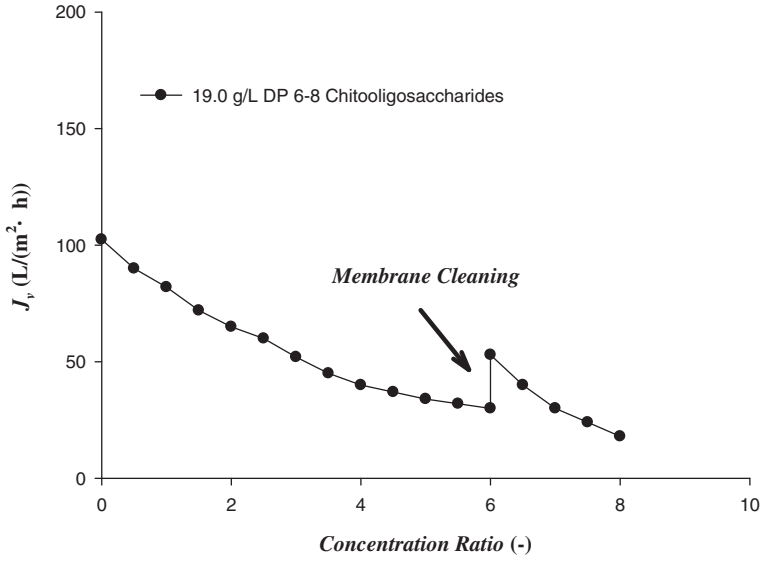

Figure 8 Concentration ratios vs. J_v of 19.0 g/L chitooligosaccharide syrup. Long-term experiments were carried out at 16.0 bar for enrichment of DP 6 to 8 chitooligosaccharides. The flux was about 102.4 L/(m^2 h) at the beginning, whereas it decreased heavily when the concentration rate was 6.0.

Purification of DP 6 to 8 COS

Long-term experiments were carried out at 16.0 bar to enrich the DP 6 to 8 COS with a virgin concentration of 19.0 g/L (pH = 5.0, V_0 = 16.0 L and T = 40°C). The performance of the NF membrane was evaluated by measuring the permeate flux and R_{obs} of the target products as illustrated in Figures 8 and 9. In addition, the concentration ratio of DP 6 to 8 COS was monitored as a formula of V_0/V_c. The flux value was about 102.4 L/(m^2 h) at the beginning of the experiment, but it decreased to 30.2 L/(m^2 h) when the concentration ratio reached to 6.0 (Figure 8). Then, the membrane was cleaned for 1 h. After that, the permeate flux of syrup was recovered to 53.5 L/(m^2 h), which indicated that about 50% of virtual flux decline due to the increasing solid content and viscosity. However, the second drop of permeate flux was remarkable till the end of the run, as the value changed from 53.5 to 25.0 L/(m^2 h) just 1.5 times of the later concentration. There was a direct reflection between the effect of solid contents and experimental records. A moderate concentration ratio and timely

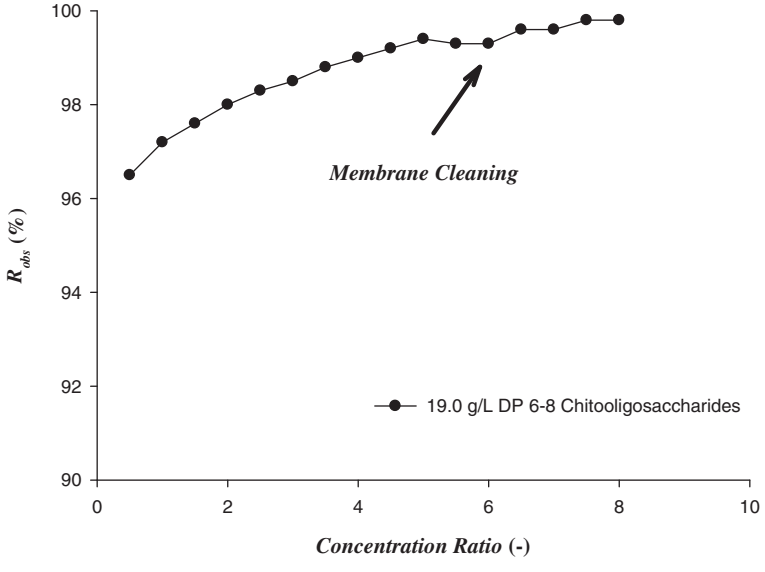

Figure 9 Concentration ratios vs. R_{obs} of DP 6 to 8 chitooligosaccharides in the syrup. The R_{obs} of DP 6 to 8 chitooligosaccharides went up when the circulating flux decreased. The phenomenon was accredited to the DSPM and steric hindrance pore (SHP) effects.

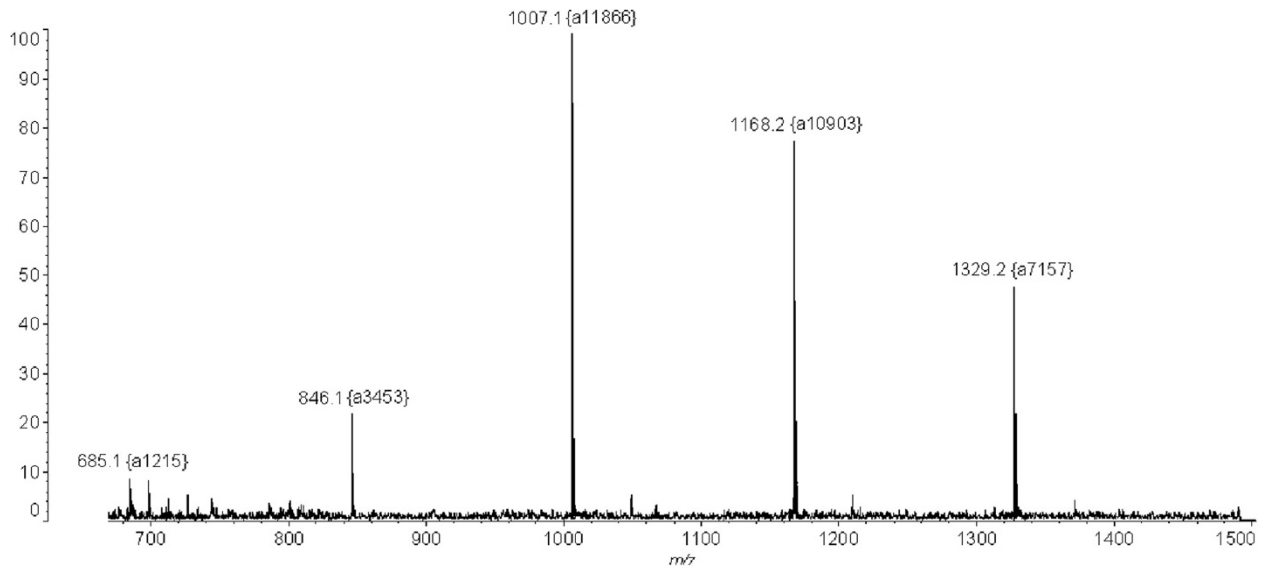

Figure 10 MALDI-TOF mass spectrum of chitooligosaccharides. It showed the distribution of chitooligosaccharides. As expected, the final products were mainly composed of DP 6 to 8 chitooligosaccharides, which was similar with the HPLC results mentioned before.

regenerations of the membrane should be arranged for acceptable efficiency in practice.

Moreover, the R_{obs} of DP 6 to 8 COS went up when the circulating flux decreased (Figure 9). The result was principally accredited to the synergy with the DSPM and steric hindrance pore (SHP) effects. On the one hand, the anions preferred to be repelled while the cations were attracted to the NF membrane. The unique property transported the amino groups, which are commonly sensed in COS, to the permeate. Ultimately, the molecules containing NH_4^+ were separated from the syrup because of the electric attraction. On the other hand, the R_{obs} of DP 6 to 8 COS was up to 1.0 after the concentration ratio increased to 8.0. The MWCO of the applied membrane is 500 Da, while the molecular weights of hexamer, heptamer, and octamer are 984, 1,145 and 1,306, respectively. There is a conspicuous difference in the sizes between membrane pores and DP 6 to 8 COS. Therefore, the observed retention behaviors illustrated that DP 6 to 8 COS were rejected sterically and a promising purity (82.2%) was eventually achieved via the NF purification designed.

MALDI-TOF-MS analysis of chitooligosaccharides

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) is an outstanding tool for the comprehensive investigation of COS because the mass spectrum can exhibit the relative quantities of a mixture to be determined. Figure 9 shows the distribution of COS using MALDI-TOF-MS after NF enrichment. As expected, the final products were mainly composed of DP 6 to 8 COS, which was similar to the HPLC results mentioned before. In the spectrum, COS often contains intensive quasi-molecular ions, which is called $[M + Na]^+$, because of its weak protonation degree. For instance, the peak at 1,007.1 m/z is attributed to the sodium form ($[M + Na]^+$) of a hexa-oligomer (Figure 10).

Conclusions

In this study, the separation behavior of COS syrups, which were enriched by different concentrations of DP 6 to 8 COS, was investigated via a bench-scale NF process.

One negatively charged membrane with MWCO of 500 Da was selected to purify DP 6 to 8 COS. During the full recycle mode, the experimental results indicated that the retentions of these components increased with pressure before 16.0 bar. Also, the operation temperature was optimized. Although the circulating flux could be improved by elevating temperature, a greater wastage of DP 6 to 8 COS was irreversibly formed during the process as well. In addition, the effects of pH on R_{obs} of COS were compared. The HPLC profiles illustrated that the R_{obs} of COS within DP ≤4 in alkali conditions was significantly higher than that in acidic environment. This phenomenon could be explained by structural curling and sterical overlap due to hydrogen bonds. However, the mechanisms should be discussed in further researches.

Under the optimum conditions (TMP = 16.0 bar, T = 40°C, and pH = 5.0), the purification of DP 6 to 8 COS was carried out. It was found that the membrane could support a reluctant flux after the concentration ratio was over 6.0 in the syrup with the concentration of 19.0 g/L DP 6 to 8 COS. MALDI-TOF mass spectrum confirmed that DP 6 to 8 COS were dominant in the final products, and the purity was up to 82.2% (w/w)

according to HPLC profiles. As a conclusion, the NF system equipped with a selected membrane module is a promising approach in the purification of DP 6 to 8 COS from specific syrups.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
This paper is the result of joint efforts. Prof. LZ designed the whole experimental plan and confirmed the main objective of this paper. Dr. YW developed the statistical methods for experimental data. HD was responsible for optimization of the nanofiltration technology and partial investigation of the transmechanism in process. QX was responsible for the quantification of proteins and total sugars. HPLC and MALDI-TOF-MS analysis were done by Prof. JZ and Prof. LJ. LF helped us complete the paper writing and correcting some grammatical errors in details. All authors read and approved the final manuscript.

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