

Rapid and Convenient Separation of Chitooligosaccharides by Ion-Exchange Chromatography

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Abstract: Pervious methods for separation of highly purified chitooligosaccharides was time-consuming and labor-intensive, which limited the large-scale production. This study developed a convenient ion-exchange chromatography using the ÄKTA™ avant 150 chromatographic system. Five fractions were automatically collected under detecting the absorption at 210 nm. The fractions were analyzed by high-performance liquid chromatography. It proved that they primarily comprised chitobiose, chitotriose, chitotetraose, chitopentaose, and chitohexaose, respectively, with chromatographic purities over 90%. The separation process was rapid, convenient and could be monitored on-line, which would be benefit for the mass production of chitooligosaccharides.

1. Introduction

Chitosan, a linear polysaccharide consisting of β (1–4) linked *N*-acetyl-*D*-glucosamine (GlcNAc) or *D*-glucosamine (GlcN) residues, is prepared from chitin by partial deacetylation. Chitosan is biodegradable, non-toxic, biocompatible, and is widely used in food, medicine, agriculture, wastewater treatment, biological materials, and other fields [1–3]. Chitooligosaccharides (COS) are the degradation oligomers of chitosan. COS have a low degree of polymerization (DP), usually $DP < 20$, leading to a high solubility in water and be easily absorbed by organisms. COS therefore show superior bioactivity over chitosan, including antioxidant [4], antimicrobial [5, 6], antitumor [7, 8], anti-inflammatory [9], and immunomodulatory [10] effects.

Using highly purified COS in bioassays is of significance to knowing which molecules are causing the effects, and even the mechanisms, of observed bioactivities. To date, however, reports on the separation of COS are scarce. Several studies have separated COS by size exclusion chromatography [11], ion-exchange chromatography [12–14], or affinity chromatography [15]. In these works, the separated COS were detected based on chromogenic reactions, such as the Imoto method, the phenol-sulfuric acid method, thin-layer chromatography (TLC), or a biconchonic acid (BCA) reagent kit, owing to the absence of chromophores of COS [7, 11–14]. For high resolution, the fractions were pooled



with a volume as small as possible and then the fractions were monitored using the above methods to draw an elution curve. Usually, hundreds of tubes of fractions were obtained, meant that hundreds of thousands of chromogenic reaction should be repeatedly done. This was time- and labor-intensive, and has limited the large-scale production of COS. This study developed a convenient ion-exchange chromatography for the separation of COS. The ultraviolet detector was used in the separation of COS. Although COS have no characteristic absorption in the ultraviolet (UV)–visible wavelength region, there are several hydroxyl and amine groups in the sugar ring. The $n \rightarrow \sigma^*$ transitions of the lone pair electrons in the heteroatoms of these groups ($-\text{OH}$, $-\text{NH}_2$) induce a strong absorption band near 200 nm. This can be used as the detection signal in the separation of COS mixtures — a mix of a homologous series. The entire separation process was rapid, convenient and could be monitored on-line, which would be benefit to industrial production.

2. Experiment

2.1 Materials

Chitooligosaccharide(COS) mixtures with a degree of deacetylation of >95% and a molecular weight of 1,500 Da were purchased from Zhejiang Golden-Shell Pharmaceutical Co., Ltd. (Zhejiang, China). Mixed chitosan oligomer standards containing chitobiose, chitotriose, chitotetraose, chitopentaose, and chitohexaose were obtained from Shanghai Hui-cheng Biological Technology Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade and purchased from Beijing Chemical Co., Ltd. (Beijing, China).

An ÄKTA™ avant 150 chromatographic system equipped with Unicorn™ 6.1 software, SP Histrap HP column (5ml), Sephadex G-10 gel packing were purchased from GE Healthcare Co., Ltd. (Uppsala, Sweden).

2.2 Preparation of Chitooligosaccharide Mixture

Low degree-of-polymerization (DP) chitooligosaccharides (LCOS) were prepared from the above COS mixtures by ethanol precipitation. First, 10 % (w/v) COS aqueous solution was added to three times the volume of ethanol. The mixture was stirred at room temperature for 4 h and kept at 4 °C overnight. Subsequently, the mixture was filtered through a Buchner to remove the insoluble high-DP oligomers; the supernatant was rotary evaporated under vacuum to remove the ethanol. The residual aqueous solution was lyophilized.

2.3 Separation of Low Degree-of-polymerization Chitooligosaccharides

The LCOS (80 mg) were dissolved in 1 mL of an acetate buffer (50 mmol/L, pH 4.6). The solution was filtered through a microporous membrane (0.45 μm) and injected into the ÄKTA™ avant 150 chromatographic system through a SP Histrap HP cation-exchange column (5ml) for separation. After washing the column with 3 column volume (cV) of HAc–NaAc buffer (50 mmol/L, pH 4.6), it was eluted with a mixture of NaCl (2 mol/L) and HAc–NaAc buffer (50 mmol/L, pH 4.6) using a linear gradient (0–60% NaCl) at a flow rate of 10 mL/min. Eluate was detected in real time by a ultraviolet (UV) detector at 210 nm and fractions were automatically collected using fraction collector according to the peak values. The start level and the end level of the peak fractionation were both 200 mAU.

The fractions were desalted by gel filtration. The fractions were loaded on a Sephadex G-10 (26 mm \times 50 cm) column and eluted with deionized water at 8 mL/min. Fractions were monitored by the UV detector at 210 nm and collected. The remaining solution was concentrated and lyophilized.

2.4 Identification of Chitooligosaccharides

HPLC analysis was performed using a Shimadzu LC-10AT_{VP} Plus instrument (Shimadzu, Kyoto, Japan) with a differential refractive-index detector. COS were separated on an Asahipak NH₂P-50E column (4.6 mm \times 25 cm) (Shodex, Shanghai, China). The optimized HPLC separation was carried out with a mobile phase consisting of acetonitrile/water (70/30, v/v) at a flow rate of 1.0 mL/min. The column

temperature was set at 25 °C.

3. Results and Discussion

3.1 Separation of Chitooligosaccharides

COS could be separated by the cation-exchange column on the basis of their differing numbers of amino groups. Under acidic conditions, the positively charged amino groups of the COS interact with the sulfopropyl ($-\text{CH}_2\text{CH}_2\text{CH}_2\text{SO}_3^-$) group of the chromatographic packing. The COS were then desorbed by the high NaCl concentration of the eluant. Longer oligosaccharide chains resulted in more amino groups on the COS and were more difficult to desorb from the column. UV detection is commonly used in separation processes; however, COS have no characteristic absorption in the UV-visible region, so the strong absorption band near 200 nm, owing to $n \rightarrow \sigma^*$ transitions of the lone pair electrons in the hydroxyl and amine groups, was used as the detection signal in the separation of the COS mixture.

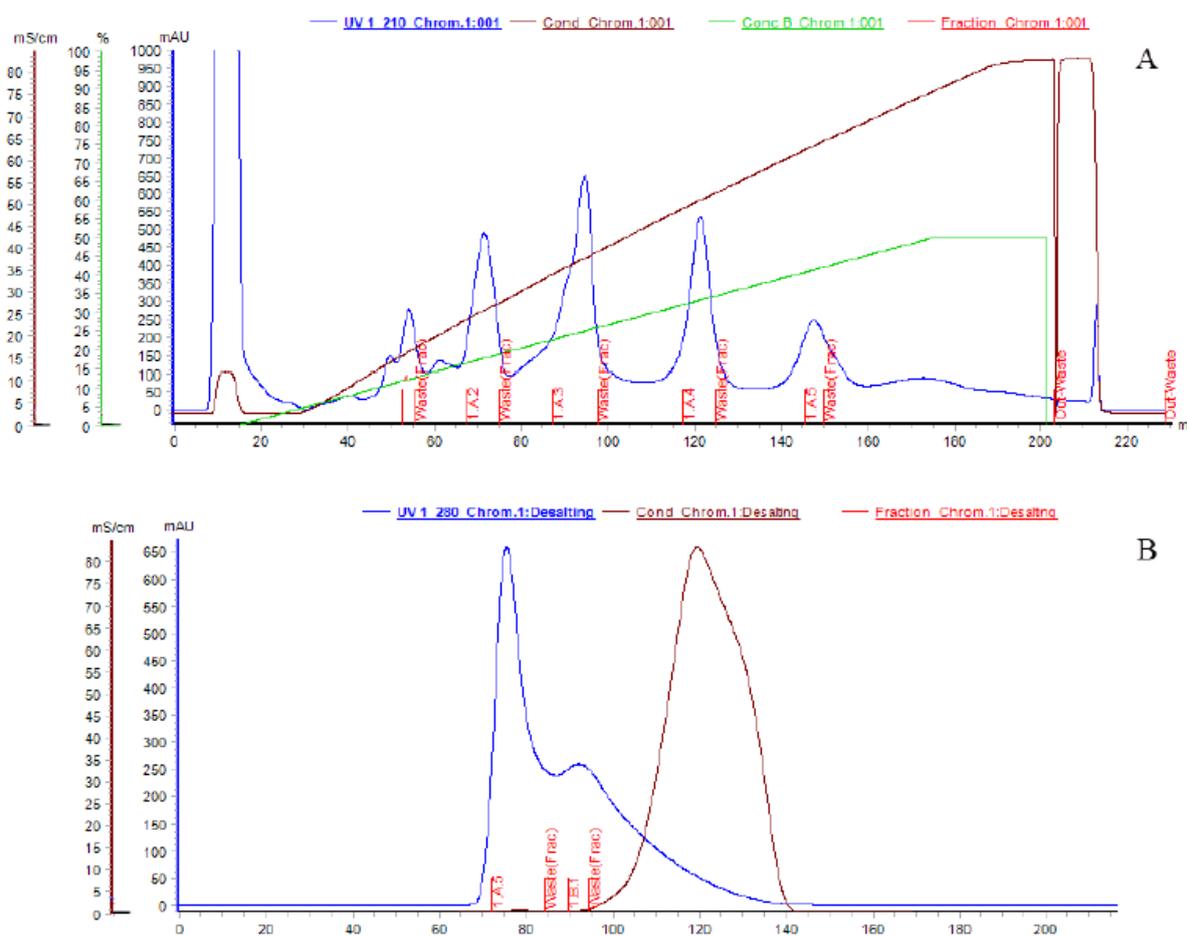


Figure 1. Chromatograms for cation-exchange via SP Histrap HP (A) and desalting via Sephadex G-10 (B). The chromatograms show the absorption signal at 210 nm in AU, the conductivity signal in mS/cm, the concentration of 2M NaCl solution in % and the fractionation collector.

The prepared LCOS were separated on the SP Sepharose HP column. Chromatograms A in figure 1 is the elution curve for cation-exchange via SP Histrap HP. The lines of different color (blue, brown and green) illustrated the absorption signal at 210 nm in AU (UV 1 210 chrom.1), the conductivity signal in

mS/cm (Cond chrom.1), the concentration of 2M NaCl solution in % (Conc B chrom.1), respectively. After washing the column, the oligomers were eluted with a mixture of NaCl and HAC–NaAc buffer. With the increase of the concentration of 2M NaCl solution, the conductivity was increase and the oligomers were desorbed. Five fractions were automatically collected when the level of the peak were higher than 200 mAU. Chromatograms B in figure 1 is the elution curve for desalting via Sephadex G-10. The results show that the conductance of each fraction was reduced to 5 mS/cm after the desalting: the NaCl in the fraction was completely removed.

3.2 Identification of the Fractions

Within a homologous series, the DP and composition of the COS can be analyzed by hydrophilic-interaction chromatography. The retention of a COS is based on the hydrophilic interactions between the stationary phase and its hydroxyl and amino groups. In general, the more hydroxyl and amino groups a COS possesses, the greater the number of hydrogen-bonding interactions with the stationary phase, i.e., the longer the oligosaccharide chain length, the longer the retention time.

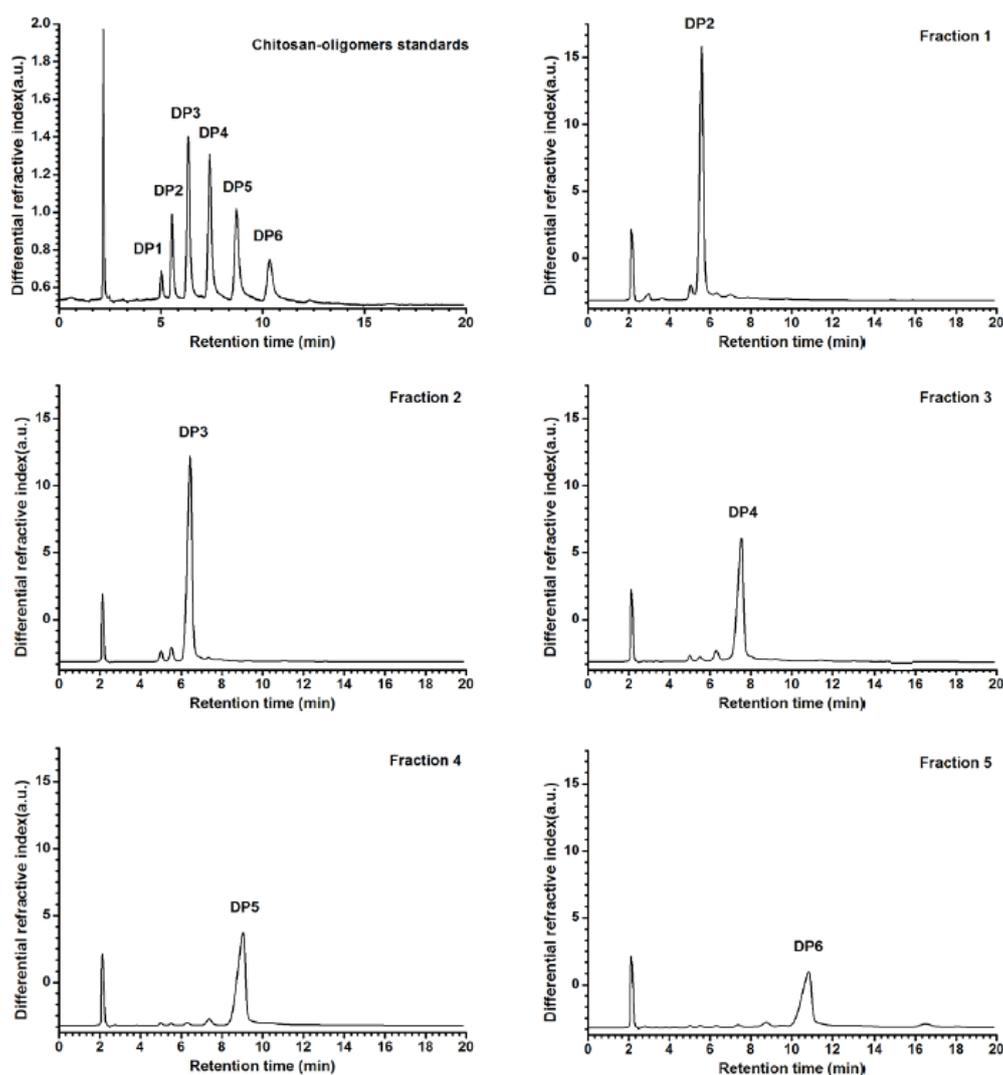


Figure 2. HPLC spectra of the chitosan oligomer standards and each separated fraction. The retention times of glucosamine di- to hexa-mers were 5.56, 6.43, 7.54, 9.01, and 10.83 min, respectively.

Table 1. The Retention Time of Chitosan-Oligomers and Fractions

Standards	glucosamine	chitobiose	chitotriose	chitotetraose	chitopentaose	chitohexaose
Retention time (min)	4.95	5.49	6.34	7.38	8.80	10.39
Fractions 1		5.56				
Fractions 2			6.43			
Fractions 3				7.54		
Fractions 4					9.01	
Fractions 5						10.83

Chitosan oligomer standards that contained chitobiose, chitotriose, chitotetraose, chitopentaose, and chitohexaose were used to calibrate the retention times. Figure 2 illustrates the DP distribution and composition of each separated fraction and its comparison with the standards. Each of the five separated fractions had an individual peak in the HPLC spectra, the retention times of which were 5.56, 6.43, 7.54, 9.01, and 10.83 min, respectively. As depicted in Table 1, these peaks matched well with those of the standards, corresponding to dimers (chitobiose), trimers (chitotriose), tetramers (chitotetraose), pentamers (chitopentaose), and hexamers (chitohexaose), respectively. Assuming that the oligomers give a similar response per mass unit, according to the relative peak areas of the HPLC spectra, the contents of the basis oligomer in fractions 1–5 were 94.87%, 91.96%, 89.11%, 91.81%, and 87.11%, respectively.

4. Conclusions

We have developed a convenient ion-exchange chromatographic method for the separation of pure COS. UV detection is used in the separation of COS. Using an ÄKTA™ avant 150 chromatographic system, five glucosamine oligomers with single DPs were isolated by ion-exchange and desalted by gel filtration. The isolated fractions contained 94.87% dimer, 91.96% trimer, 89.11% tetramer, 91.81% pentamer, and 87.11% hexamer, respectively. The entire separation process is rapid, convenient and could be monitored on-line, which would be benefit to industrial production.

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6. References

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