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Separation of chito-oligomers with several degrees of polymerization and study of their antioxidant activity

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ABSTRACT

Chitooligomers (COS) with degree of polymerization (DP) ranging from 2 to 12 was prepared by acid hydrolysis of chitosan and five fractions were separated from the prepared COS by CM Sephadex C-25 column. The fractions were desalted by activated charcoal extraction. The components of each fraction were analyzed by TLC, HPLC and MALDI-TOFMS. Five fractions mainly contained trimer (95.7%), tetramer and pentamer (90.9%), pentamer (85.6%), hexamer (89.5%) and heptamer to decamer (94.7%), respectively. The antioxidant activities of each fraction and COS mixture were investigated, including hydroxyl and superoxide radical scavenging activity and reducing power. The activities of the six COSs were in a dose-dependence manner and related to their DP. The COS with low DP showed better effect of scavenging hydroxyl radical and reducing power than that with high DP. In contrast, the superoxide radical scavenging activity of all the tested COSs increased with DP increasing.

1. Introduction

Chitooligomers (COS) is the hydrolysate of chitosan mainly made up of β-1,4 linked D-glucosamine (GlcN) and partially of β-1,4 linked N-acetyl-D-glucosamine (GlcNAc). Previous study showed that COS possessed a huge potential in medicine and food fields due to its wide bioactivity, such as antitumor activity, radical scavenging, antimicrobial activity, immunity modulatory effect and wound healing (Aam et al., 2010; Muzzarelli, 2009; Xia, Liu, Zhang, & Chen, 2011). At present, large amounts of COS can be obtained by acidic hydrolysis, oxidative degradation, microwave, γ-irradiation or by enzymatic hydrolysis (Kang, Dai, Zhang, & Chen, 2007; Muzzarelli, Terbojevich, Muzzarelli, & Francescangeli, 2002; Trombotto, Ladaviere, Delolme, & Domard, 2008; Xing, Liu, Yu, et al., 2005). All the techniques applied to prepare COS produce the mixtures containing COS with different molecular weights. Most of reported bioactivities of COS were also assayed using mixtures. It is difficult to know which COSs with well-defined DP play a significant role in the bioactivity assay. For further bioactivity studies, the separation of COS with very narrow molecular weight was required.

Toshiaki et al. employed capillary electrophoresis to separate and monitor COS with two to six saccharides chains (Hattori, Anraku, & Kato, 2010). Size exclusion chromatography or ultrafiltration membrane was applied to realize the separation of COS in previous studies (Heggset et al., 2010; Jeon & Kim, 2000). However, these methods need expensive equipment and do not allow for large-scale preparation of COS with narrow DP. Frantz et al. separated several COSs with single DP by immobilized metal affinity column chromatography but the tetramer was the upper limit of efficient separation in this system (Ledevedec et al., 2008).

Ion-exchange chromatography was developed with great sample load, fast flow and high resolution, which have been widely applied in the separation of biomolecules (Levison, 2003). Chitooligomers was one natural cationic sugar with free amino groups. In acid buffer, protonated amino groups interacted with the ion-exchange material. It was possible that COS with different DP was separated according to their numbers of amino groups. Xiong et al. (2009) prepared several COSs with single DP by ion-exchange resin (Dowex 50WX8-200). Wei, Wang, Xiao, and Xia (2009) utilize ion-exchange column chromatography to separate one component mainly consisting of chitohexaose. However, the separation conditions of ion-exchange chromatography still need to be optimized in order to obtain more single DP COSs with high purity. In addition, a mass of salt in the eluted solution produced great operational difficulties for subsequent desalination of COS, especially for COSs with DP lower than 6 due to small difference in molecule weight between COS and salt. The desalination methods of COS with low DP remained to be further explored.

The present study describes a separation method of COS based on ion-exchange column chromatography. Activated charcoal extraction was developed to desalt the separated fractions. Five fractions were obtained, which mainly contained trimer, tetramer

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and pentamer, pentamer, hexamer and heptamer to decamer, respectively. The antioxidant activity of COS had been reported by prior researchers, whereas the antioxidant activity of each individual COS was unknown. For this reason, the antioxidant activity of each separated fraction was studied.

2. Experiment

2.1. Materials

Commercial α-chitosan from shrimp shell, with degree of deacetylation of 82% and molecular weight of 658 kDa, was purchased from Qingdao Baicheng Biochemical Corp. (China). Five standard COSs, glucosamine dimer (≥98%), glucosamine trimer (≥96%), glucosamine tetramer (≥97%), glucosamine pentamer (≥90%), and glucosamine hexamer (≥80%), were obtained from Dalian Glycobio Co., Ltd. (Dalian, China). Glucosamine hydrochloride, Darco G-60 activated charcoal (100 mesh), nitrotetrazolium blue chloride (NBT), phenazine methosulfate (PMS) and nicotinamide adenine dinucleotide-reduced (NADH) were purchased from Sigma Chemicals Co. All other chemicals and reagents were analytical grade.

2.2. Preparation of chitooligomers

COS was prepared by acid hydrolysis of chitosan. Briefly, chitosan (10 g) was added to 300 mL HCl solution (6 mol/L), which was heated in a 70 °C bath under stirring for 2 h. The hydrolyzate was adjusted to pH 8–9 with concentrated NaOH and subsequently filtered through a Buchner funnel to remove insoluble parts and the filtrate was concentrated on a rotary evaporator and precipitated by adding four-fold volume of ethanol at 4 °C overnight. The precipitate was collected by centrifugation for 15 min at 4000 rpm then lyophilized to yield powdered products and referred as COS-HCl.

2.3. Separation of COS by CM Sephadex C-25

COS-HCl (800 mg) was dissolved in 10 mL of HAc–NaAc buffer (50 mmol/L, pH 4.8), and then filtered with a microporous membrane (0.45 μm) to obtain a clear solution. The filtrate was loaded on a CM-Sephadex C-25 (50 cm × 2.6 cm) column. Later on, the column was eluted with HAc–NaAc buffer (50 mmol/L, pH = 4.8) and different concentrations of NaCl (0–2 mol/L)–HAc buffer stepwise at 3 mL/min. Fractions were collected and monitored by the phenol–sulfuric acid method at 490 nm (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.4. Desalination of fractions by activated charcoal extraction

The collected fractions were stirred with activated charcoal Darco G-60 (100 mesh) for 30 min to remove NaCl. This mixture was filtered through a filter paper under vacuum, and the oligosaccharides adsorbed onto the activated charcoal were desorbed by stirring in 50% aqueous ethanol for 30 min. Activated charcoal was eliminated by filtering, and the solution was evaporated under vacuum. $AgNO_3$ aqueous solution was applied to monitor whether NaCl was completely removed. The desalination operation was repeated until NaCl was absent. The final solution was filtered through a 0.45 μm cellulose acetate membrane and lyophilized.

2.5. TLC analysis of COS fractions

Fractions were deposited on thin layer chromatography (TLC) silica gel plates and separated using a mobile phase made up of n-propanol, water, ammonia water (7:3:1, V/V/V). After migration, pots were visualized with a solution of ethanol containing 0.1% of ninhydrin sprayed on the plate and heated at 100 °C for 15 min.

2.6. HPLC and MALDI-TOF MS analysis of COS

The isolated fractions were analyzed by hydrophilic interaction/reversed-phase chromatogram using an Agilent 1260 series HPLC system (Agilent, USA) with evaporative light scattering detector (Alltech, USA) (Guo et al., 2009, 2007). Chromatography was performed on a Click Maltose column (4.6 mm × 150 mm, 5 μm), using binary mobile phases (acetonitrile and ammonium formate buffer) stepwise at a flow rate of 1.0 mL/min with column temperature at 30 °C.

The MALDI-TOF mass spectrometry was measured on a Biflex III Bruker mass spectrometer (Germany) in a positive ion mode employing 2,5-dihydroxybenzoic acid (DHB) as a matrix. For ionization, a nitrogen laser (337 nm) was used.

2.7. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay was carried out according to Guo et al. (2005). The reaction mixture, total volume 4.5 mL, containing the samples of COS, was incubated with EDTA–Fe^{2+} (220 μM), safranine O (0.23 μM), H_2O_2 (60 μM) in potassium phosphate buffer (150 mM, pH 7.4) for 30 min at 37 °C. The absorbance of the mixture was measured at 520 nm. Hydroxyl radicals bleached the safranine O, so increased absorbance of the reaction mixture indicated the sample had strong hydroxyl radical scavenging activity and the capability of scavenging hydroxyl radical was calculated using the follow equation:

$$\text{Scavenging effect}\,(\%) = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100$$

where A_{blank} was the absorbance of the blank (distilled water instead of the samples), A_{control} was the absorbance of the control (distilled water instead of H_2O_2).

2.8. Superoxide radical scavenging assay

The superoxide radical scavenging ability of COS was assessed by the method of Nishikimi, Appaji Rao, and Yagi (1972). The reaction mixture, containing COS, PMS (30 μM), NADH (338 μM), and NBT (72 μM) in Tris–HCl buffer (16 mM, pH 8.0), was incubated at room temperature for 5 min and the absorbance was measured at 560 nm against a blank. The capability of scavenging superoxide radical was calculated using the following equation:

$$\text{Scavenging effect}\,(\%) = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

where A_{control} was the absorbance of the control (Tris–HCl buffer instead of NADH).

2.9. Measurement of reducing power

The reducing power of COS was determined by the method described earlier by Yen and Chen (1995). Briefly, 1.25 mL of reaction mixture, containing different concentration of COS in phosphate buffer (0.2 M, pH 6.6), was incubated with 1.25 mL potassium ferricyanide (1%, W/V) at 50 °C for 20 min. The reaction was terminated by 2.5 mL trichloroacetic acid solution (10%, W/V). Then the solution was mixed with 1.5 mL ferric chloride (0.1%, W/V) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

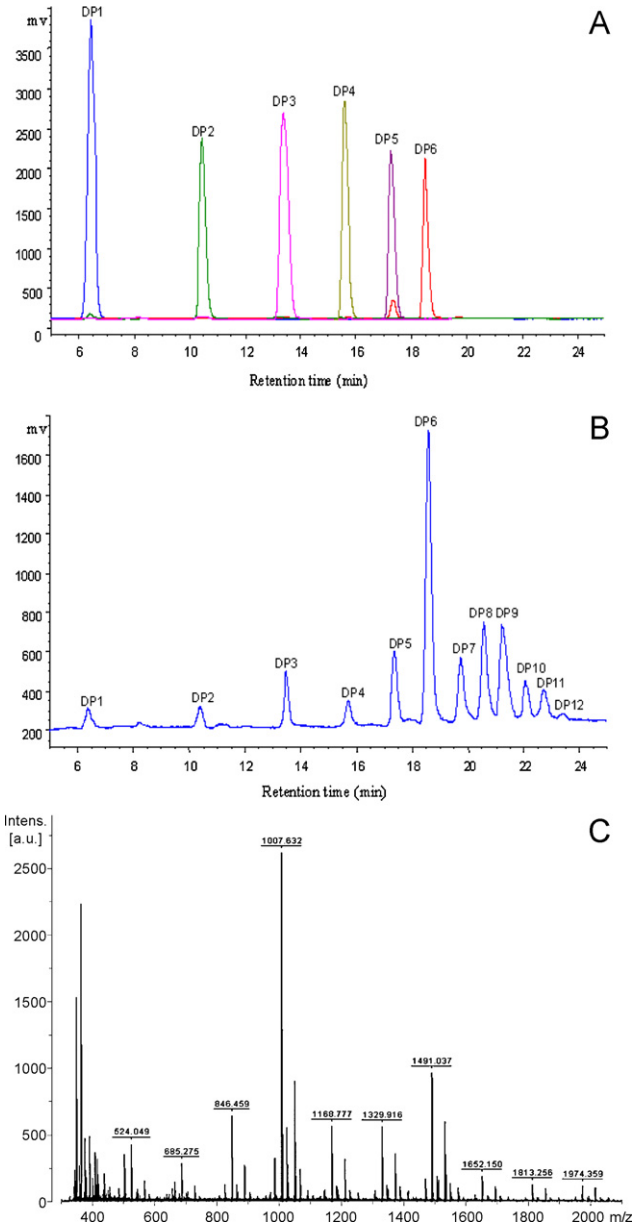

Fig. 1. HPLC and MALDI-TOF MS spectrum of COS. (A) HPLC spectrum of glucosamine oligomers standards (glucosamine monomers, glucosamine dimer, glucosamine trimer, glucosamine tetramer, glucosamine pentamer and glucosamine hexamer). (B) HPLC spectrum of COS-HCl. (C) MALDI-TOF MS spectrum of COS-HCl. Ten peaks were all [M+Na$^+$] ion-peaks of glucosamine oligomers with 161 Da mass larger than the peak ahead, which was the exact molecular mass of a GlcN residue.

2.10. Statistical analysis

The data presented were means ± S.D. and followed by Duncan's multiple-range test. Differences were considered to be statistically significant if $P < 0.05$.

3. Results

3.1. Preparation of chitooligomers

As shown in Fig. 1, the prepared COS (COS-HCl) was analyzed by HPLC and MALDI-TOF MS. Fig. 1A showed the retention times of monosaccharide (glucosamine hydrochloride) and five glucosamine oligomers standards (glucosamine dimer, glucosamine trimer, glucosamine tetramer, glucosamine pentamer and glucosamine hexamer) were 6.5, 10.4, 13.4, 15.7, 17.3 and 18.5 min, respectively. The components of COS-HCl were illustrated in Fig. 1B. There were twelve fractions detected in our HPLC system. Compared with the retention time of glucosamine oligomers standards, the dimer, trimer, tetramer, pentamer and hexamer existed in COS-HCl. A small peak at 6.5 min showed that COS-HCl contained little glucosamine. Hence, it was speculated that the six peaks after 18.5 min corresponded to COS with DP 7–12. Fig. 1C showed the MALDI-TOF MS spectrum of COS-HCl, which indicated similar results with HPLC. It was clearly observed that ten peaks corresponding to the glucosamine oligomers with DP 3–12 were monitored in the spectrum, while monomer and dimer were not detected due to interference of matrix (below 500 m/z). The mass/charges (m/z) of the main peaks were 524.049, 685.275, 846.459, 1007.632, 1168.777, 1329.916, 1491.037, 1652.150, 1813.256, and 1974.359, respectively. Ten peaks were all [M+Na$^+$] ion-peaks with 161 Da mass larger than the peak ahead, which was exactly the molecular mass of a GlcN residue. To summarize, this analysis displayed that the distribution of COS-HCl ranged from 2- to 12-mers and centered on DP 6, and COS-HCl could be used as materials for the preparation of COS with single DP.

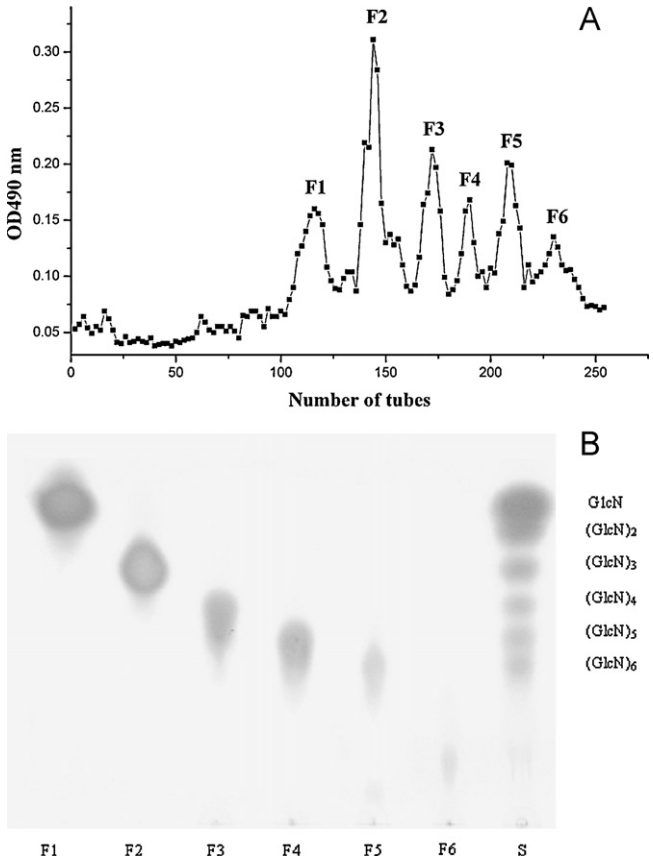

Fig. 2. Chromatographic profile of COS-HCl on CM Sephadex C-25 column (A) and thin layer chromatography analysis of fractions (B).

3.2. Separation of COS by CM Sephadex C-25

Fig. 2 depicted the chromatographic profile of COS-HCl on a CM Sephadex C-25 column and TLC analysis of each fraction, which provided a preliminary insight of separation in the collected fractions. Six distinct, well-separated fractions, according to amino group numbers of COS, were collected in our operating conditions and then desalted and lyophilized. Glucosamine and glucosamine oligomers (DP 2–6) could be separated by TLC. It was evidently

Table 1
Components of five fractions analyzed by HPLC and MALDI-TOFMS.

Fractions	Retention time (min)	m/z[a]	Components	Content (%)[b]
F2	13.4	524.049	$(GlcN)_3$	95.7
F3	15.7	685.246	$(GlcN)_4$	90.9[c]
	16.3	888.439	$(GlcN)_4GlcNAc$	
F4	17.3	846.368	$(GlcN)_5$	85.6
F5	18.5	1007.360	$(GlcN)_6$	89.5
F6	19.6	1168.553	$(GlcN)_7$	94.7[c]
	20.4	1329.670	$(GlcN)_8$	
	21.2	1532.806	$(GlcN)_8$ GlcNAc	
	22.0	1693.899	$(GlcN)_9$ GlcNAc	

[a] The mass/charge corresponding to $[M+Na^+]$.
[b] Calculated as the percentage of peak area in HPLC spectrum.
[c] Total content of listed COS. The content of each COS could not be exactly calculated because baseline separation was not achieved.

revealed that each fraction had main component except F6 which was not extended due to DP > 6. TLC showed that the main component of F1 was the unwanted glucosamine monomer, and therefore F1 would be absent in the following research.

3.3. Analysis of fractions on HPLC and MALDI-TOFMS

F2, F3, F4, F5 and F6 were analyzed by HPLC (Fig. 3) and MALDI-TOFMS (Fig. 4), which allowed the distribution of COS in each eluted fraction. As is shown in Fig. 3, the retention time of main peak in F2 matched well with glucosamine trimer standard. The purity of glucosamine trimer in F2 was 95.7%, according to the percentage of peak area in HPLC spectrum. The MALDI-TOFMS spectrum showed the main mass/charge (m/z) of F2 was 524.049 corresponding to $[M+Na^+]$ ion-peak of glucosamine trimer. In the same manner, the analytic results of all the fractions were summarized in Table 1. The purities of glucosamine pentamer in F4 and glucosamine hexamer in F5 were 85.6% and 89.5%, respectively.

3.4. Hydroxyl radical scavenging activity of each fraction and COS-HCl

The scavenging activities of each fraction and COS-HCl at different concentrations towards hydroxyl radical produced by Fenton reaction were shown in Fig. 5. All the samples exhibited obvious scavenging activity against hydroxyl radical in a concentration-dependent manner. The hydroxyl radical scavenging activity curve of F2 lifted visibly compared to other COSs, which indicated it showed the highest activity. No obvious difference was observed among F3, F4 and F5. The hydroxyl radical scavenging activities of F2, F3, F4, F5, F6 and COS-HCl were 48.19, 44.46, 44.42, 43.83, 34.97and 40.40% at 3.33 mg/mL, respectively. It suggested that the order of the ability to scavenge hydroxyl radical was F2 > F3, F4, F5 > COS-HCl > F6. Hydroxyl radical scavenging activity of COS increased with DP decreasing.

3.5. Superoxide radical scavenging activity of each fraction and COS-HCl

Fig. 6 showed that COS-HCl and five fractions exhibited dose-dependent superoxide radical scavenging activity. Among the six COSs, F6 showed the highest capacity of scavenging superoxide radical at all the tested concentrations. The concentration at which 50% of the superoxide radicals were scavenged, IC_{50} values of F2, F3, F4, F5, F6 and COS-HCl were 1.30, 1.30, 1.29, 1.11, 0.68 and 0.89 mg/mL, respectively. The scavenging effect of COSs decreased in the order of F6 > COS-HCl > F5 > F4, F3, F2. These results indicated that COS with high DP had stronger ability to scavenge superoxide radical.

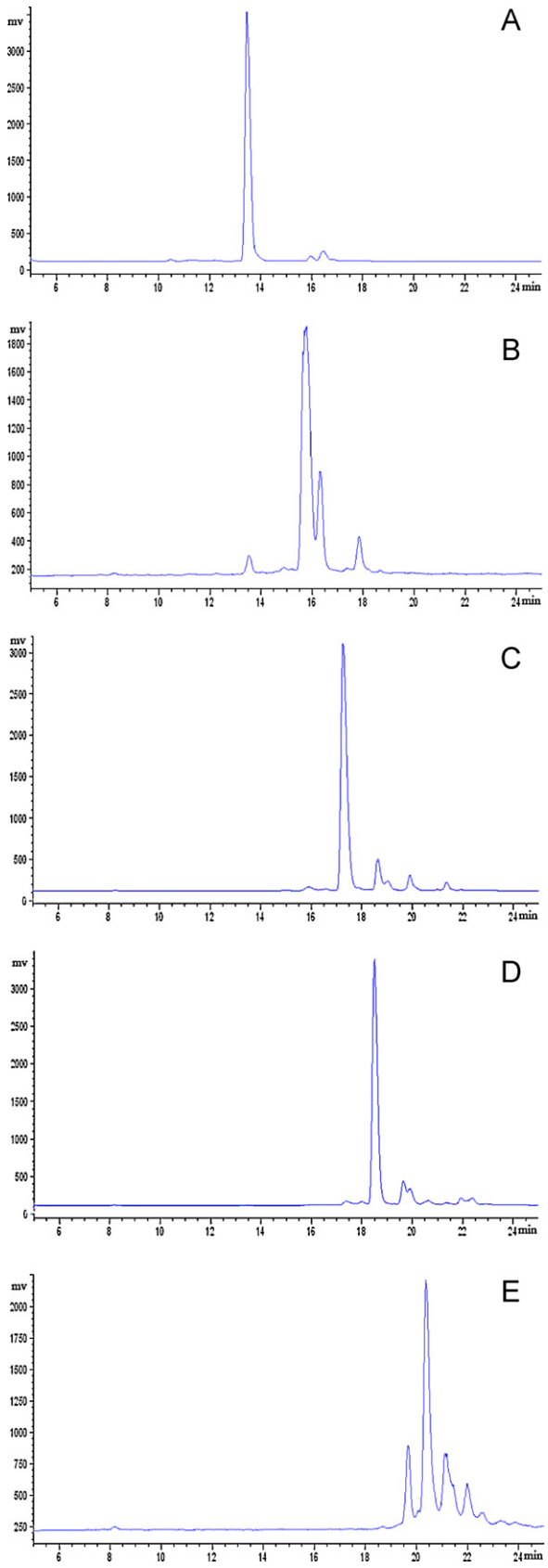

Fig. 3. HPLC spectrums of F2 (A), F3 (B), F4 (C), F5 (D) and F6 (E) on Click Maltose column. (A) One main peak with the retention time of 13.4 min. (B) Two main peaks with the retention time of 15.7 and 16.3 min. (C) One main peak with the retention time of 17.3 min. (D) One main peak with the retention time of 18.5 min. (E) Four main peaks with the retention time of 19.6, 20.4, 21.2, 22.0 min.

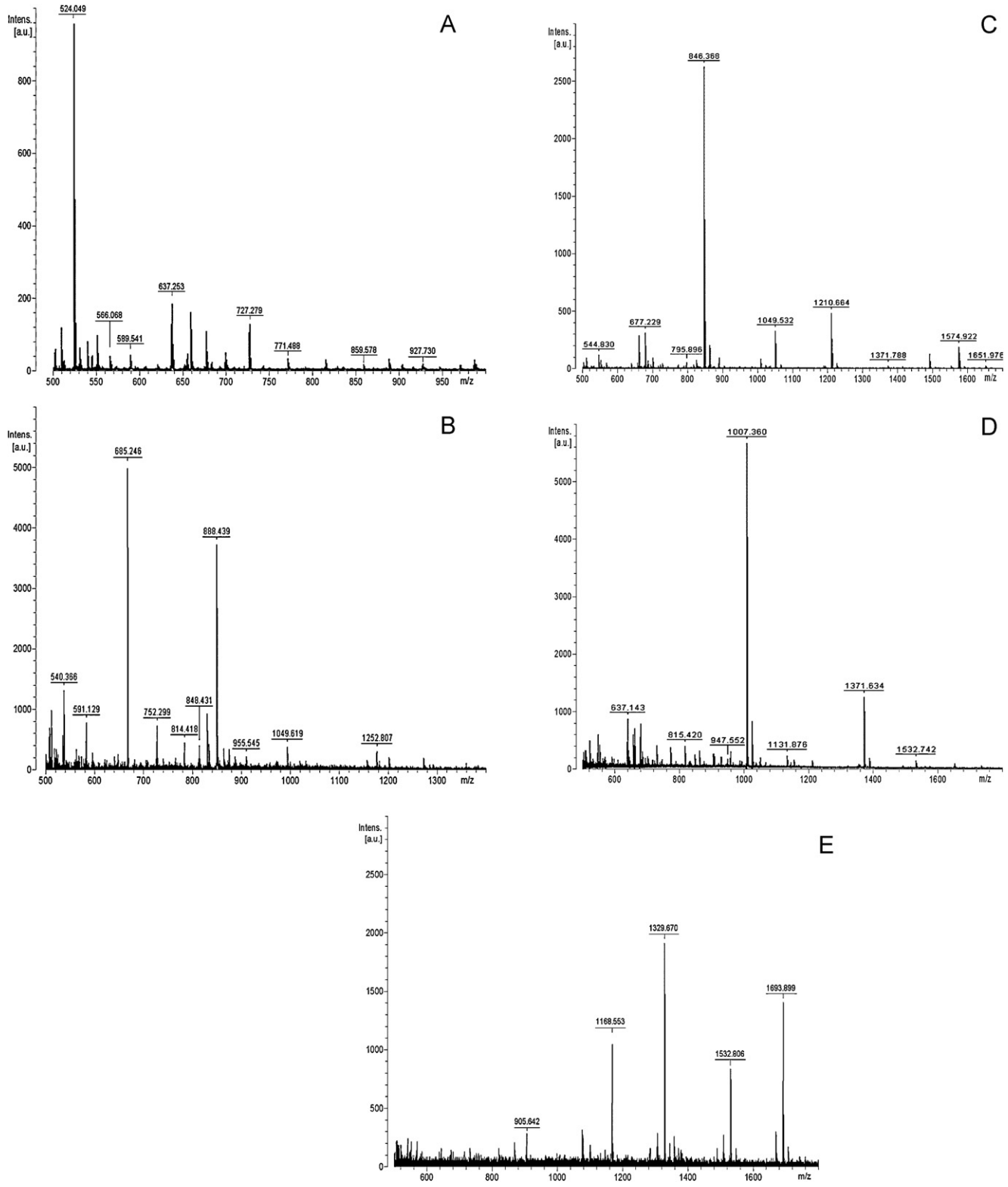

Fig. 4. MALDI-TOFMS spectrums of F2 (A), F3 (B), F4 (C), F5 (D) and F6 (E). All the main peaks correspond to the mass charge of their [M+Na$^+$].

3.6. Reducing power of each fraction and COS-HCl

Fig. 7 depicts the reducing power of all the tested samples. The reducing power of all kinds of COSs correlated well with increasing concentrations. Only one fraction, F2 exhibited more pronounced reducing power than COS-HCl. The reducing powers of F2 and COS-HCl at 0.78 mg/mL were 0.572 and 0.451. Moreover, F3, F4, F5 and F6 showed similar reducing power.

4. Discussion

In the ion-exchange column chromatography process, no oligomers were eluted using HAc–NaAc buffer in the first 50 tubes

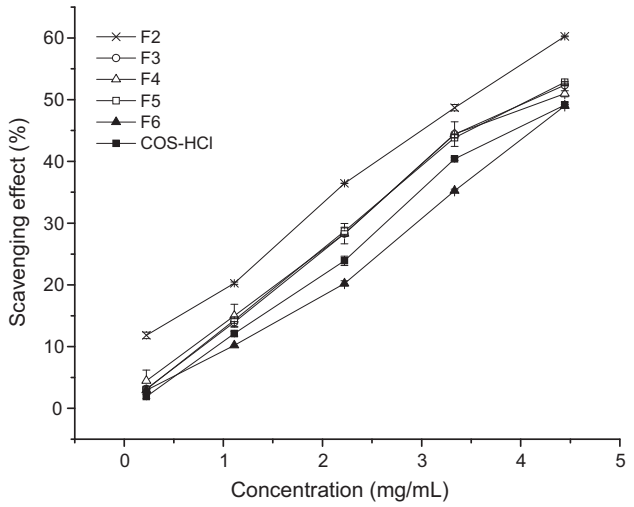

Fig. 5. Scavenging effect of each fraction and COS-HCl on hydroxyl radicals. Results are mean ± standard deviation of three parallel measurements.

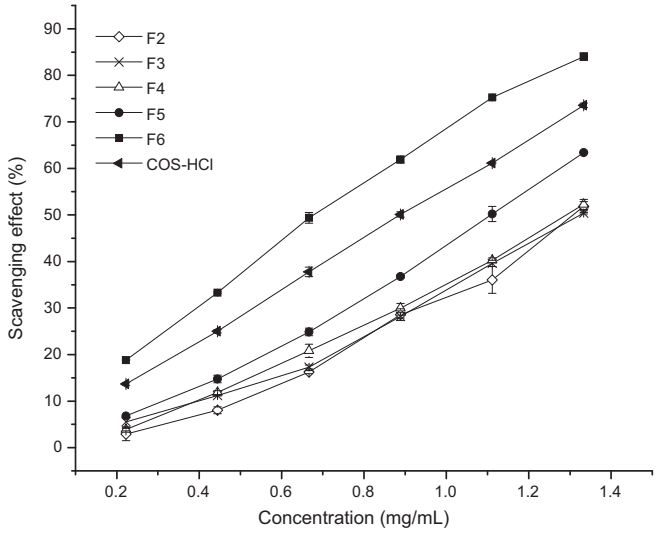

Fig. 6. Scavenging effect of each fraction and COS-HCl on superoxide radicals. Results are mean ± standard deviation of three parallel measurements.

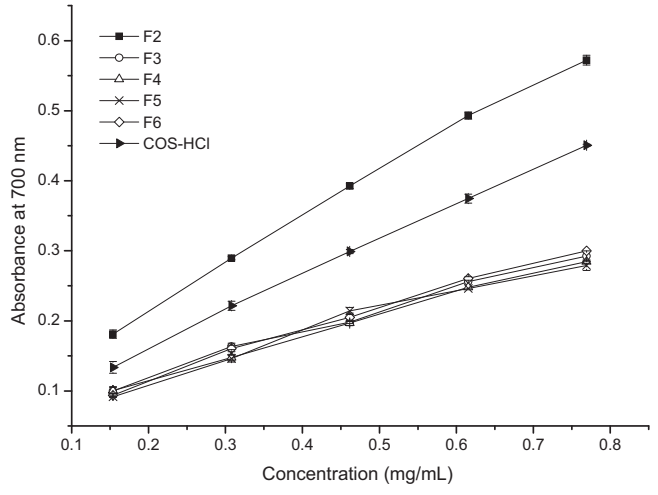

Fig. 7. Reducing power of each fraction and COS-HCl. Results are mean ± standard deviation of three parallel measurements.

(more than one column volume). Therefore, the COSs (800 mg) loading onto ion-exchange column were all absorbed and it was possible, under this high loading which was two times of what Wei, Wang, Xiao, and Xia (2009) reported, to separate each COS. Three different acidic buffers (pH 3.6, 4.8 and 5.8) were tested since COS was positively charged only in acidic aqueous solution. The retention time became shorter with pH value decreasing. The short retention time meant COS was eluted at low concentration of NaCl, which was cost-effective and beneficial to desalination. However, the resin volume was compressed seriously at pH 3.6. Therefore, the middle pH value (4.8) was selected for separation of COS. Flow rate was an important factor that could influence the separation of COS. Earlier tests with low flow rates did not allow well separation. At a flow rate of 3 mL/min, we achieved well separation of COS. The cationic resin was regenerated by 3 bed volumes buffer through the column. The separation method was assayed for a dozen times which show well intra-laboratory reproducibility.

The desalination of the fractions was performed by activated charcoal extraction. The conventional desalination methods like gel chromatography and dialysis are time-consuming and have difficulty in desalting of oligosaccharides with M_w < 1000 Da. Activated charcoal is a common sorbent which could absorb polar molecules in polar solution and desorb them in apolar solution. Oligosaccharides contain plenty of hydroxyl groups which could form hydrogen bond interaction with activated charcoal and are absorbed. The interaction becomes stronger with the chains of oligosaccharides increasing, which could be applied to separation of oligosaccharides. Wei, Hendrix, and Nieman (1997) isolated two oligosaccharide fractions enriched in tetra- and pentasaccharides from honeydew employing charcoal column chromatography. Fujimoto, Hattori, Uno, Murata, and Usui (2009) utilized a column of charcoal-celite to separate synthesized gentiooligosaccharides and obtained four main fractions (DP 2–5). Moreover, Yang, Zhao, He, and Croft, (2010) reported that they removed monomer in galactooligosaccharides employing activated charcoal extraction due to the weak interaction between monomer and activated charcoal. Similar to the oligosaccharides mentioned above, COS should also interact with activated charcoal and be absorbed with little monomer. Considering that activated charcoal hardly absorbs inorganic salt, we presumed that desalination of COS by activated charcoal extraction was feasible. COS could be captured by activated charcoal while NaCl still stayed in water, and then the desalination of COS was achieved. There was not precipitation in the desalted fractions behind adding $AgNO_3$ aqueous solution, which confirmed that all the fractions used in the following study were pure COSs without NaCl.

HPLC and MALDI-TOFMS were employed to analyze the distribution of COS in each eluted fraction. Three COSs with high purity were obtained and the purity of glucosamine hexamer was as high as 89.5%, which was purer than that of glucosamine hexamer standards. Nevertheless, the components of F3 and F6 were complex. The mass spectrum demonstrated that the key components of F3 were glucosamine tetramer and mono-N-acetyl chitopentaose and F6 mainly consisted of four chitooligomers with DP from 7 to 10. Although N-acetyl chitooligomers in F3 and F6 were not quantified due to imperfect baseline separation, we speculated that the proportion of N-acetyl chitooligomers was low (below 30%) according to peak area in HPLC. It was noteworthy that the free amino group numbers of glucosamine tetramer and mono-N-acetyl chitopentaose in F3 were both 4, whereas those of F2, F4 and F5 were 3, 5 and 6, respectively. The total content of glucosamine tetramer and mono-N-acetyl chitopentaose in F3 was 90.9%. Therefore, it is possible that glucosamine tetramer with high purity (>90%) could be separated by our method if fully N-deacetylated COS was used as materials. However, the main components of F6 were four COSs with different free amino groups, which indicated that it was

difficult to separate those COSs with DP ≥ 7 by this method. F6 could be further separated by combining other techniques that was underway in our laboratory.

The bioactivity of COS with well-defined DP has attracted wide interest, but there are few reports about the bioactivity of the separation fractions of COS mixture. In this case, we separated five fractions from the prepared COS mixture by CM Sephadex C-25 column and investigated antioxidant activity of each fraction.

Previous studies reported COS exhibited high antioxidant activity, such as radical scavenging in vitro and inhibiting oxidative stress in cells (Je, Park, & Kim, 2004; Liu et al., 2009; Ngo, Kim, & Kim, 2008; Yuan, 2009). It had been found that the antioxidant activity of COS was significantly related to average molecular weight (M_w) (Kim & Thomas, 2007; Park, Je, & Kim, 2003). However, the effect of DP of COS on antioxidant activity was unknown. In the present study, the antioxidant activities of five COS fractions with different DP were investigated employing various established in vitro systems, including hydroxyl and superoxide radical scavenging activity and reducing power.

Our data demonstrated that the scavenging hydroxyl radical activity of COS increased with DP decreasing. Glucosamine trimer showed the highest hydroxyl radical scavenging activity among the test COS. Previous researches showed that scavenging hydroxyl radical activity of COS was significantly related to its average molecular weight. Our team had reported that the antioxidant activity of chitosan and its derivatives became strong with molecular weight declining (Xing, Liu, Guo, et al., 2005; Zhong et al., 2007). Sun et al. studied antioxidant activity of four COSs with different molecular weight (2300, 3270, 6120, and 15,250 Da) and obtained consentaneous results (Sun, Zhou, Xie, & Mao, 2006). Chen et al. found that two kinds of COSs with the lowest Mw, chitobiose and chitotriose were more potent than glucosamine hydrochloride and three reference compounds (aminoguanidine, pyridoxamine and a water-soluble α-tocopherol analogue) in scavenging hydroxyl radical (Chen et al., 2003). Our results further confirmed that COS with low DP had better hydroxyl radical scavenging activity. Compared with chitosan, COS has short chains and weak intramolecular and intermolecular hydrogen bond. Therefore, the free hydroxyl and amino groups are activated, which help to promote antioxidant activity. However, the presence of more than 2 saccharides chains in chitooligomers is important for the hydroxyl radical scavenging activity.

In this case, COS with high DP showed stronger ability to scavenge superoxide radical than that with low DP. Like hydroxyl radical scavenging activity, the superoxide radical scavenging activity of chitosan and COS also had been reported to increase with M_w decreasing in above-mentioned literature. However, superoxide radical scavenging activity of COS with low DP (M_w < 3000 Da) had been revealed in a different rule. Yang, Shu, Shao, Xu, and Gu (2005) found that COS with 1100 Da had higher superoxide radical scavenging activity than COS with 500 Da using the same method. Park et al. (2003) reported that COS (M_w 3000–1000 Da) showed stronger superoxide radical scavenging activity than COS (M_w 1000 Da). In combination to prior studies, our results suggested that there existed optimal COS with very narrow DP for superoxide radical scavenging activity. However, superoxide radical scavenging mechanism was not clear and remained to further research.

The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron. In this case, only glucosamine trimer among all the test fractions exhibited more pronounced reducing power than COS-HCl. Our team had studied the reducing power of glucosamine hydrochloride which was stronger than that of chitotriose at all the tested concentration (Xing et al., 2006). As a result, we inferred that COSs with DP ≤ 3 played a leading role in the reducing power.

5. Conclusion

It was concluded as follows: (1) According to the analytical results by TLC, HPLC and MALDI-TOFMS, we thought that the separation method of chitooligomers based on ion-exchange column chromatography was feasible. Three highly purified chitooligomers with single DP (glucosamine trimer 95.7%, glucosamine pentamer 85.6% and glucosamine hexamer 89.5%) were obtained and the purity of glucosamine hexamer was higher than that of glucosamine hexamer standards (80%). In order to obtain the better separation effect, other separation conditions are being explored. (2) The antioxidant activity of chitooligomers was significantly related to its DP. The chitooligomers with low DP showed better effect of scavenging hydroxyl radical and reducing power than that with high DP. (3) The superoxide radical scavenging activity of all the tested chitooligomers increased with DP increasing.

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