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Carbohydrate Polymers 72 (2008) 701–706
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Preparation and properties of reduced chitooligomers

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Received 9 August 2007; received in revised form 21 October 2007; accepted 23 October 2007
Available online 1 November 2007

Abstract

In order to prevent the browning of the chitooligomer product prepared from enzymatic hydrolysis, the chitooligomers were reduced by potassium borohydride. The chemical structures and physical properties of the reduced chitooligomers were characterized by magnetic resonance spectra, X-ray diffraction, UV-spectrophotometry, thermogravimetric analysis, differential thermal analysis. The chemical stability of the chitooligomers increased after reduction. The reduced chitooligomers had no oral acute toxicity, and they had almost the same inhibition effect against the sarcoma 180 tumor cells in mice by intraperitoneal injection as the fresh chitooligomers.

Keywords: Chitosan; Chitooligomer; Stability; Reduction; Antitumor activity

1. Introduction

Chitooligomers are expected to be utilized for functional food and medical supplies because of their many beneficial biological properties (Harish Prashanth & Tharanathan, 2007; Kim & Rajapakse, 2005), including their antitumor activity (Harish Prashanth & Tharanathan, 2005; Jeon & Kim, 2002; Liang, Chen, Yen, & Wang, 2007; Maeda & Kimura, 2004; Qi & Xu, 2006; Qin, Du, Xiao, Li, & Gao, 2002; Seo, Pae, & Chung, 2000; Suzuki et al., 1986; Tokoro et al., 1998), immuno-enhancing effects (Kobayashi, Watanabe, Suzuki, & Suzuki, 1990; Peluso et al., 1994; Tokoro et al., 1988), antibacterial activity (Choi et al., 2001; Jeon, Park, & Kim, 2001; No, Park, Lee, & Meyers, 2002), enhancing protective effects against infection with certain pathogens (Tokoro, Kobayashi, Tatewaki, Suzuki, & Okawa, 1989).

However, chitooligomers have short shelf life because they have a lot of reducing units (Zeng et al., 2007). As a consequence, the shorter the chain of chitooligomer, the higher the probability that aldehyde groups react with amino groups. These Schiff, Maillard and Amadori reactions impart the chitooligomer product undesirable properties such as brown color, scarce solubility and loss of physiological activity. The treatment of chitooligomers with ammonia, which was an alternative to the chitosan amino groups, prevents insolubility, dark coloring and short shelf life of the product, but the reports on their biological activities had not been found (Muzzarelli, Terbojevich, Muzzarelli, & Francescangeli, 2002).

This work was concerned with the properties of the reduced chitooligomers. KBH_4 was used to reduce chitooligomer product prepared by enzymatic hydrolysis of chitosan. The reduced chitooligomers product was comparatively investigated by NMR, UV, TGA/DTA and X-rays diffraction. The two products were also used to assay their antitumor activity against sarcoma 180 in mice.

2. Experimental

2.1. Materials

Initial chitosan CS0 (DDA 81%) and the crude hemicellusase solution were from Hubei Yufeng Biology Engineering Co. Ltd. (China). The enzyme was purified according our previous paper (Qin et al., 2002). The UF membranes

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doi:10.1016/j.carbpol.2007.10.014

(OSOO1C11, OMEGA) with NMWL10 and 1 kDa were purchased from PallFiltron Corporation (USA). The pullulan standard series (MW 47,300, 22,800, 11,800, 5900) was TOSOH products. A chitooligomer standard series from dimmer to hexamer were purchased from Seikagak Corporation (Japan).

2.2. Characterizations

Weight–average molecular weight (M_w) was measured by GPC. The GPC equipment consisted of column TSK G3000-PW, TSP P100 pump and RI 150 refractive index detector. The flow rate was maintained at 1.0 ml/min. 0.2 M CH_3COOH/0.1 M CH_3COONa was used as the eluent. The pullulan and chitooligomer standard series were used to calibrate the column. All data provided by the GPC system were collected and analyzed using the Jiangshen Workstation software package.

NMR spectra were recorded on a Varian Mercury Vx300 spectrometer at ambient temperature. The samples were dissolved in D_2O.

MALDI-TOF-MS analysis of chitooligomers was carried out using Voyager DE STR matrix assisted laser desorption ionisation time-of-flight mass spectrometry (Applied Biosystems, USA) at an acceleration voltage of 20 kV using 2,5-dihydroxybenzoic acid as the matrix.

UV absorption spectra were obtained using a Shimadzu 1601 UV–vis spectrophotometer.

X-ray diffraction patterns of samples were measured by a Shimadzu XRD-6000 diffractrometer and used a CuKα target at 40 kV and 30 mA at 23 °C.

TG and DTA of sample (5.0 mg) in stationary air were performed by a Differential Thermal Analyzer Model WCT-1 (Beijing Optical Instruments Factory, China) from 25 to 450 °C at a heating rate of 10 °C/min.

2.3. Preparation of chitooligomer sample (COS) and reduced chitooligomer sample (R-COS)

Chitosan (100 g) was completely dissolved in 2000 ml 2% acetic acid. The solution in reaction vessel was placed in a water bath at 50 °C and 10 ml enzyme solution was added to initiate the reaction. After 4 h, UF-membrane of 10 kDa was used to remove enzyme, and the UF-membrane of 1 kDa was used to separate the product.

The half of the ultrafiltrate through the UF-membrane of 1 kDa was concentrated under diminished pressure. The mixtures were neutralized to pH 9 and precipitated by adding ethanol. The precipitates were washed thoroughly with ethanol. The precipitates were collected after drying over phosphorus pentoxide *in vacuum* to get the sample COS.

The other half of the ultrafiltrate through the UF-membrane of 1 kDa was neutralized to pH 7. KBH_4 (2 g) was added into the solution, and the mixture was stirred at room temperature for 24 h. The mixture was neutralized to pH 9, and precipitated by adding 2000 ml ethanol. The precipitates were washed thoroughly with ethanol. The precipitates were collected after drying over phosphorus pentoxide *in vacuum* to get the white powder R-COS.

2.4. Oral acute toxicity in mice

Kunming strain mice (4 weeks old) weighing 20–24 g were purchased from Hubei Experimental Animal Center (China). Prior to dosing, they were fasted overnight. The animals were divided into four groups of five males and five females, and R-COS was administered by oral gavage at doses of 1000, 2150, 4640 and 10,000 mg/kg body weight. The observation of general status, toxic symptom and mortality in rats was continued for 7 days after treatment. Finally, the maximum tolerant dose and acute toxic classification were determined.

2.5. Assays of antitumor activity

Antitumor effect on sarcoma 180 was observed in normal Kunming mice (five male and five female/each group). The test was made by observing the effect on the growth of the tumor in ascite form of a dose of 0.2 ml (about 1.0×10^7/ml) implanted subcutaneously at the right groin. After 24 h of the tumor implantation, the test sample dissolved in physiological saline was provided once a day by intraperitoneal injection (i.p.) for 10 days, while 0.9% saline was provided for the control group. The animals were sacrificed, and the tumors were dissected and weighted. The tumour growth inhibition ratio was calculated by using the formula: *Inhibition rate* $(\%) = 100 \times (C - T)/C$, where C is the average tumor weight of the control group and T is the tumor weight of the treated sample groups. Mean values and SD were determined by standard methods with the SPSS program, and the significance of difference was estimated by the standard Students t-test.

3. Results and discussion

3.1. Gel permeation chromatography of the degraded product

The chitosan was efficiently hydrolysed by hemicellulase to obtain chitooligomers. The GPC of chitosan and its degraded product were shown in Fig. 1. After 4 h for

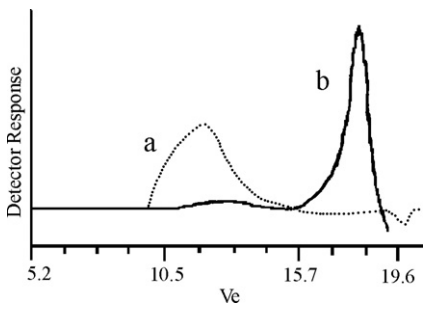

Fig. 1. GPC of initial chitosan and its degraded product.

hydrolysis, the main peak of degraded product is between peaks of standard $(GlcN)_5$ and $(GlcN)_6$, suggesting that the main components in the degraded product were chitooligomers. The M_w of separated COS sample is 1.5×10^3.

3.2. MALDI-TOF-MS analysis of COS

Chitooligomers have been shown to exhibit similar signal strengths, irrespective of structure, when examined by MALDI-TOF-MS. Therefore, MALDI mass spectrum allows the relative quantities of constituents of a mixture to be determined. Fig. 2 shows the MALDI-TOF-MS spectrum of COS. The result revealed that the products were composed mainly of chitooligomers, especially of degree of polymerisation (DP) 3 to 8. The chitooligomers with the same DP also contained many different molecules, which had different numbers of acetyl group in different positions.

3.3. NMR spectra

The chitooligomer COS was reduced by KBH_4 to obtain the reduced chitooligomer product R-COS, which also had good water-solubility. Fig. 3 shows the ^{13}C NMR spectra of fresh COS and R-COS in D_2O. The six strong signals at 57.0, 60.7, 74.5, 75.2, 78.8 and 102.9 ppm attributed to C-2, C-6, C-3, C-5, C-4 and C-1 on the bone residues, respectively. The assignments of signals are based on data found in the literatures (Fukamizo, Ohtakara, Mitsutomi, & Goto, 1991; Saitô, Mamizuka, Tabeta, & Hirano, 1981). The C-1 peaks of α and β-anomers around 90 and 95 ppm in R-COS obviously disappeared in comparison with COS.

The weak signals of H-1(α) and H-1(β) in the ^1H NMR spectrum also disappeared after COS was reduced to R-COS (data not shown). These results indicated that the reducing units were modified.

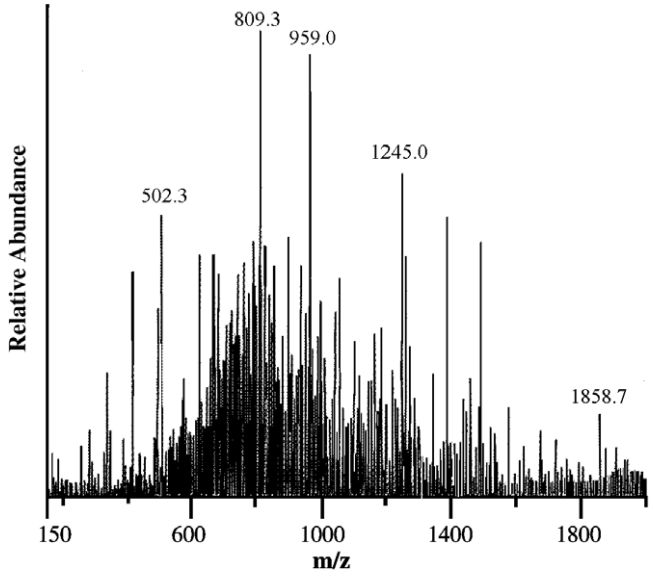

Fig. 2. MALDI-TOF-MS spectrum of COS.

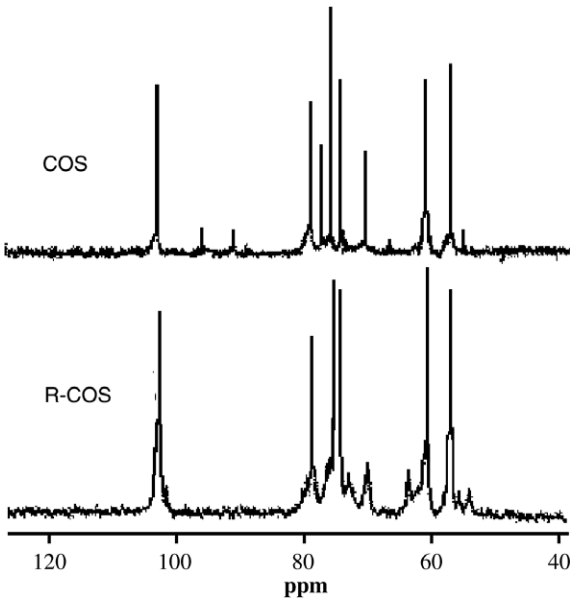

Fig. 3. ^{13}C NMR spectra of COS and R-COS in D_2O.

3.4. X-ray analysis

Fig. 4 shows the X-ray diffraction patterns of resulting samples. The COS mainly shows the "annealed" type pattern, which was identified by the three characteristic diffraction peaks at $2\theta = 15.2°$, 21.2° and 23.7° (Ogawa, 1991; Saitô & Tabeta, 1987). After the reducing end was reduced, the crystallinity sharply decreased, and the R-COS only had a decreased wide peak at $2\theta = 19.8°$ and became amorphous structure (Cervera et al., 2004). Chitooligomers after oxidation of the reducing ends also became amorphous structure (Qin, Du, & Xiao, 2002).

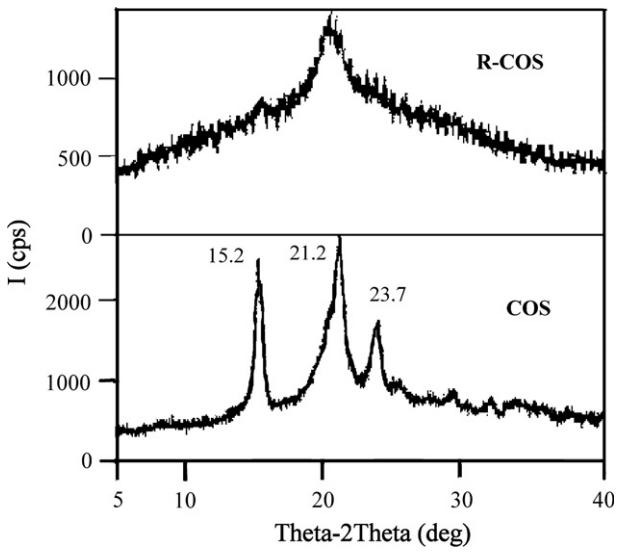

Fig. 4. X-ray diffraction patterns of COS and R-COS.

These results indicated that the reducing ends are very important for the preservation of crystal structure in chitooligomers.

3.5. Thermal analysis

The TG and DTA curves of COS and R-COS in static air are shown in Fig. 5. Both samples had a weight loss before 140 °C, which was accompanied by first endothermic effects as the result of evaporation of adsorbed and bound water. As is known, polysaccharides usually have a strong affinity for water and hydration properties (Kittur, Prashanth, Sankar, & Tharanathan, 2002). The water molecules are associated with hydrophilic amine groups and hydroxyl groups in chitosan. The weight loss of COS was faster than that of R-COS.

The DTA curves show that COS started to decompose at around 160 °C, and had several peaks before 350 °C. The degradation of COS was composed of a set of concurrent and consecutive reaction (Kim & Lee, 1993; Qu, Wirsén, & Albertsson, 2000). The exothermic effect before 250 °C might come from the reaction of reducing end residues in the small molecules of COS. The maximum pyrolysis around 293 °C for COS was due to partial breaking of the main chain and the disintegration of intramolecular interaction. Then, there was an exotherm, which was likely due to further decomposition of degraded fragments, charring and oxidation of the products. In the above process, the dehydration and the evaporation of degradation products had endothermic effect. The endothermic and exothermic reactions could occur at the same time so that the expected endotherm might be overwhelmed by the following overlapping exotherm with partial compensation. The degradation process seems to be so complex that a detailed explanation is too difficult (Kittur et al., 2002), and this should be further investigated.

R-COS started to decompose after 200 °C, and had lower exothermic effect before 300 °C than COS. The results indicated that R-COS was more thermally stable than COS.

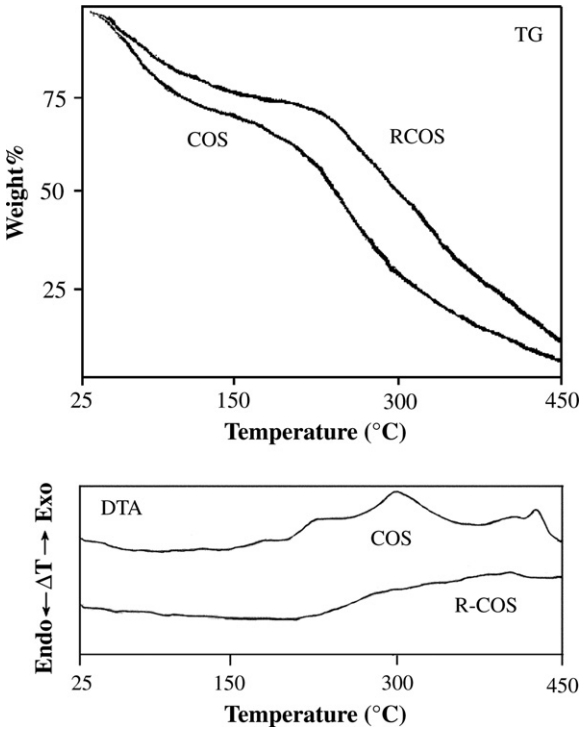

Fig. 5. TG and DTA curves of COS and R-COS.

3.6. Stability of the chitooligomer solutions

Fig. 6 shows the UV-absorption spectra of solution of 1% COS and 1% R-COS after heating. After heating at 100 °C for 10 min, COS solution turned brown, which had very stronger absorption than R-COS in the test wavelength range.

3.7. Oral acute toxicity in mice

Mice administered R-COS did not develop any clinical signs of toxicity either immediately or during the post-treatment period even at the highest dose of 10 g/kg body weight. The general conditions of all mice were normal. No mortality occurred either immediately or during the 7-day observation period. The oral maximum tolerant dose of COS was more than 10 g/kg body weight in mice. Therefore, the oral acute toxicity in mice was no toxicity according to the criteria of acute toxic classifications.

3.8. Antitumor activity

Table 1 shows that the chitooligomers COS and R-COS significantly reduced the sarcoma 180 tumor growth in male and female mice when treated by intraperitoneal injection. Both COS and R-COS had higher tumour growth inhibition ratio on the female mice than the male mice at a dose of 50 mg/kg, suggesting that the female mice might be more sensitive to the chitooligomers than the

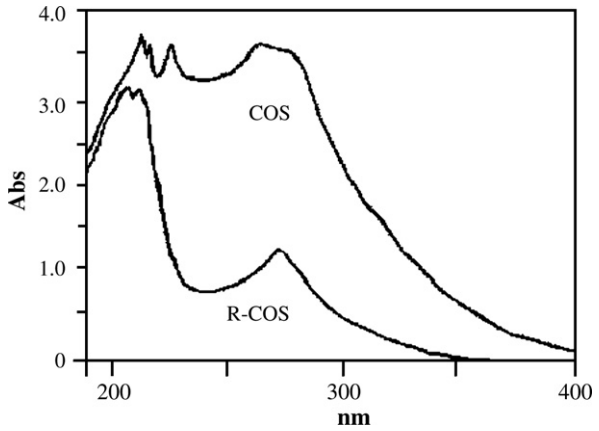

Fig. 6. UV spectra of 1.0% COS and R-COS solution after heating at 100 °C for 10 min.

Table 1
Effect of chitooligomers on sacroma 180 solid tumor growth

Mice	Sample	Dose (mg/kg/day)	Body weight (g)		Tumor weight (g)	Inhibition (%)
			Initial	Final		
Male	Control		18.98 ± 1.11	25.68 ± 2.70	2.00 ± 0.19	
	COS	50	19.80 ± 0.863	29.65 ± 2.81	1.42 ± 0.46*	29.0
		200	19.68 ± 0.832	29.16 ± 2.27	1.18 ± 0.49*	41.0
	R-COS	50	19.86 ± 0.93	30.66 ± 2.36	1.38 ± 0.46*	31.0
		200	18.66 ± 0.577	26.88 ± 0.59	1.28 ± 0.49*	36.0
Female	Control		18.10 ± 0.60	23.78 ± 1.00	2.08 ± 0.70	
	COS	50	18.64 ± 0.67	24.18 ± 2.42	1.02 ± 0.20*	51.0
		200	19.10 ± 1.16	26.18 ± 3.13	1.40 ± 0.56	32.7
	R-COS	50	18.54 ± 0.67	27.56 ± 3.35	1.16 ± 0.28*	44.2
		200	19.76 ± 1.04	27.46 ± 1.16	1.56 ± 0.53	25.0

* $P < 0.05$.

male mice. The tumour growth inhibition ratio had no significant difference on the same sexual mice between COS and R-COS at the same dose. The amino groups might contribute a lot to the antitumor effect of chitosan (Muzzarelli, 1977). The low M_w chitosan with oxidized reducing-end also had the similar antitumor activity as the chitooligomrs (Qin et al., 2002). The chitosan could undergo the enzyme-catalyzed degradation in the body to become smaller molecules (Onishi & Machida, 1999). Thus, the reducing ends in chitosan sample might be not very important for the antitumor activity.

Hexa-*N*-acetylchitohexaose and chitohexaose were reported to be growth-inhibitory against S180 and MM-46 solid tumors transplanted into male mice when given by i.v. administration (Suzuki et al., 1986). The hexaoses were also growth-inhibitory to Meth-A solid tumor transplanted in male mice, but the lower homologs of the hexaoses were unable to exhibit the same effect. The antitumor mechanism of chitosan were assumed to be acceleration of the production of and response to interleukin-1 and interleukin-2 for maturation of splenic T-lymphocytes to killer T-cells, but the antitumor mechanism of hexa-*N*-acetylchitohexaose was different from that of chitohexaose (Tokoro et al., 1988). The water-soluble chitosan with MW around 3 kDa had better antitumor activity on the male and female mice than the chitooligomrs and macromolecular chitosan (Jeon & Kim, 2002; Qin, Du, Xiao, Li, & Gao, 2002). Seo et al. (2000) reported that the synergism between the effects of interferon (IFN)-γ and water-soluble chitosan on nitric oxide (NO) synthesis and tumoricidal activity was dependent mainly on the increased secretion of tumor necrosis factor-α induced by water-soluble chitosan. Maeda and Kimura (2004) suggested that antitumor activity of low-molecular-weight chitosan might be due in part to the enhancement of natural killer activity of intestinal intraepithelial lymphocytes in mice.

4. Conclusions

The R-COS had lower crystallinity but higher chemical stability than COS. The R-COS had no oral acute toxicity, and it had the similar antitumor activity as the fresh COS. These results suggest that the reduction of chitooligomers both improve the shelf life and preserve the biological activities. Thus, the reduced chitooligomers have promising applications in food for human health.

Acknowledgments

This work was partially supported by Natural Science Foundation of China (20472066), and Natural Science Foundation of Hubei (2006ABB039). We thank Mr. Gao X.H., Ms. Zhou J.L. and Liu H.L. for their help with antitumor experiments.

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