Bioorganic & Medicinal Chemistry Letters 21 (2011) 798–800

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Antioxidant activity of *N*-acyl chitosan oligosaccharide with same substituting degree

Tao Sun *, Yun Zhu, Jing Xie, Xuhong Yin

College of Food Science & Technology, Shanghai Ocean University, Shanghai 201306, China

ARTICLE INFO

Article history:
Received 17 June 2010
Revised 31 October 2010
Accepted 20 November 2010
Available online 9 December 2010

Keywords:
Chitosan oligosaccharide (COS)
N-Acyl chitosan oligosaccharide
Antioxidant activity
Substituting degree

ABSTRACT

N-Maleoyl chitosan oligosaccharide (NMCOS) and *N*-succinyl chitosan oligosaccharide (NSCOS) were prepared by acylation with maleic anhydride and succinic anhydride, respectively. Their structural changes were confirmed by Fourier-transform infrared (FT-IR) spectroscopy and their substituting degrees were determined both as 0.49 by conductometric titration. Their antioxidant activities were evaluated by scavenging superoxide anion O_2^-, hydroxyl radical ·OH and determination of reducing power. The 50% inhibition concentrations (IC_{50}) of NMCOS and NSCOS scavenging effect on O_2^- were 2.25 and 3.27 mg/mL, respectively. The IC_{50} of NMCOS scavenging effect on ·OH was 0.24 mg/mL, however, at the same concentration determined, the value of NSCOS on ·OH was 30.5%. The reducing powers of NMCOS and NSCOS at the concentration of 2.40 mg/mL were determined as 0.46 and 0.41, respectively. The above results showed that NMCOS has better antioxidant activities, which may be related to the fact that maleoyl has stronger electron-withdrawing effect than succinyl.

Chitosan, which is a copolymer consisting of β-(1→4)-2-acetamido-D-glucose and β-(1→4)-2-amino-D-glucose units, is derived from chitin by deacetylation in the alkali condition. Compared with chitosan, chitosan oligosaccharide (COS) has better water solubility and intrinsically hypoallergenic. It is suitable for systemic transport in an effective and essentially harmless manner; this might lead to outcome with a clinical interest.[1] It has been reported that COS showed various functional properties including lowering blood cholesterol, lowering of high blood pressure, protective effects against infections, antimicrobial effects, controlling arthritis and enhancing antitumor.[2–6] Recently antioxidant activity of chitosan oligosaccharides and its derivatives has attracted much attention due to their nontoxic nature and natural abundance.[7,8] Acylation of chitosan is an important modification method to prepare chitosan derivatives with good water solubility, biocompatibility and unique bioactivities.[9–11] However, there was little report on the antioxidant activity of *N*-acyl chitosan oligosaccharides. In order to research the relationship between the properties of acyl groups and antioxidant activity of *N*-acyl chitosan oligosaccharides, *N*-maleoyl chitosan oligosaccharide (NMCOS) and *N*-succinyl chitosan oligosaccharide (NSCOS) with the same substituting degree were synthesized and their antioxidant activities were investigated.

Chitosan oligosaccharide (5.0 g, 7310 Da, supplied by Zhejiang Jinke Biochemistry Co., Ltd) was dissolved in water (100.0 mL), then maleic anhydride solution (1.5 g in 50.0 mL acetone) was added and stirred for 15 h at room temperature. The resultant solution was added into three-amount acetone to cause a precipitation, then refined three times by the dissolving–precipitating process. The precipitation was collected and dried under vacuum at 60 °C for 24 h to obtain NMCOS.[12]

N-Succinyl chitosan oligosaccharide was synthesized by the modified method of Zhu Aiping.[13] Five grams of COS was dissolved in water (100.0 mL), then succinic anhydride solution (2.5 g in 50.0 mL acetone) was added drop wise for 30 min and stirred for 4 h at 40 °C. The resultant solution was precipitated in acetone and washed with acetone repeatedly, then dried under vacuum at 40 °C for 24 h to obtain NSCOS.

The structural changes of *N*-acyl COS were confirmed in a Fourier-transform infrared (FTIR) spectrometer (Nicolet NEXUS 470). Both FT-IR spectra of NSCOS and NMCOS showed the characteristic absorption bands of COS. For NSCOS the absorption of new peak at around 1400–1200 cm^{-1}, corresponding to the carboxyl group of the succinyl, which indicated COS was substituted by succinic anhydride.[13] NMCOS's new peak appeared at 1630 cm^{-1} corresponding to double bond of the maleoyl group, which indicated that maleic anhydride had been introduced into COS.[14] In addition, scarcely any changes in band at 1038 and 1071 cm^{-1} were assigned to the stretching vibration of the primary and secondary –OH group, respectively, which suggested that the oxygen atoms in the hydroxyl group in COS were not involved in the reaction.[15,16] These results indicated that NSCOS and NMCOS were obtained.

The substitution degrees of NMCOS and NSCOS were measured by conductometric titration according to the following procedure.[17] NMCOS and NSCOS were dissolved in 20.0 mL HCl solution (0.1038 mol/L) and diluted with 200 mL deionized water, then ti-

* Corresponding author. Tel.: +86 21 61900363; fax: +86 21 61900365.
E-mail address: taosun@shou.edu.cn (T. Sun).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.11.097

trated by NaOH solution (0.4685 mol/L). According to the titration volume of NaOH and the electric conductivity, the substitution degrees of NMCOS and NSCOS were calculated as 0.49 and 0.49, respectively.

Superoxide anion scavenging activity was evaluated by chemiluminescence technology on a bio-chemical luminometer (IFFDM-D, Xi'an, China).[18] Superoxide anion was produced by a luminol-enhanced autoxidation of pyprogallol. The chemiluminescent reaction was processed in a Na_2CO_3–$NaHCO_3$ (pH = 10.20, 0.05 mol/L) buffered solution at room temperature. The final concentration of luminol was 4×10^{-4} mol/L and pyprogallol was 5×10^{-5} mol/L. Scavenging activity of the samples was evaluated according to their quenching effects on the chemiluminescence signal of the luminal-pyrogallol system. The capability of scavenging against superoxide anion was calculated as: Scavenging effect (%) = $(CL_0 - CL_1)/CL_0 \times 100\%$, where CL_0 and CL_1 represent chemiluminescence peak areas of the blank group and test group, respectively. The free radical produced in the system was proved to be superoxide radical tested by superoxide dismutase, catalase and mannitol.

Hydroxyl radical scavenging activity was processed as a similar program described above. Hydroxyl radical was produced in a Fe(II)-H_2O_2-luminol system. The chemiluminescent reaction was processed in K_2HPO_4–NaOH (pH = 7.4, 0.05 mol/L) buffered solution at room temperature. The final concentrations were: $K_4Fe(SCN)_6$ 0.8 mg/mL, H_2O_2 0.012 mol/L and luminol 6.4×10^{-4} mol/L. Scavenging activity of the samples was evaluated according to their quenching effects on the chemiluminescence signal of the system. The capability of scavenging against hydroxyl radical was calculated as: Scavenging effect (%) = $(CL_0–CL_1)/CL_0 \times 100\%$, where CL_0 and CL_1 represent chemiluminescence peak areas of the blank group and test group, respectively. The hydroxyl radical was proved by SOD, catalase and mannitol.

The reducing power of the samples was determined by the method of Yen and Chen.[19] Different concentrations of the samples solutions (2.0 mL) were mixed with 2.5 mL sodium phosphate buffer (pH 6.60, 0.2 M) and 2.5 mL potassium ferricyanide (1% W/V). The mixtures were incubated for 20 min at 50 °C, and then 2.5 mL trichloroacetic acid (10% W/V) was added to the mixtures, followed by centrifugation at 2000 rpm for 10 min. The supernatant was mixed with 2.5 mL distilled water and 0.5 mL ferric chloride solution (0.1% W/V) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Data of antioxidant evaluation were expressed as mean ± standard error of the mean (n = 3) and independent student's t-test was used to determine the level of the significance (Originpro 6.1, P <0.05).

Superoxide anion shows as a relatively weak oxidant and exhibits limited chemical reactivity, but it can generate dangerous species, including singlet oxygen and hydroxyl radicals, which will cause the peroxidation of lipids.[20] In the present study, superoxide anion scavenging effect of NMCOS and NSCOS at different concentrations was showed in Figure 1. Scavenging effect of superoxide anion was evident at all the tested concentrations. IC_{50}'s of NMCOS and NSCOS were estimated as 2.25 and 3.27 mg/mL, respectively. This result showed that NMCOS had higher activity upon the elimination of superoxide radical compared to NSCOS. In this system, the superoxide anion scavenging effect of COS was better than that of NMCOS and NSCOS at all the tested concentrations, and the IC_{50} of COS was 1.24 mg/mL. Ji et al. had reported that superoxide anion is zwitterionic radical.[21] It could react with free hydroxyl and amino groups in chitosan and its derivatives to form a most stable macromolecular radical. The antioxidant activity of Schiff base of chitosan and carboxymethyl chitosan reduced because the amino and hydroxyl groups were substituted.[22] Similarly, in this antioxidant evaluation system COS had better superoxide anion scavenging effect compared with NMCOS and NSCOS because no active groups were substituted.

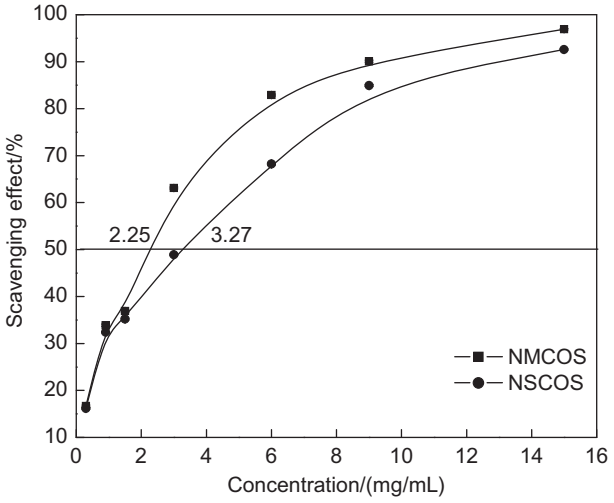

Figure 1. Scavenging effects of NMCOS and NSCOS on superoxide anion.

The hydroxyl radical is considered the most reactive free radical in biological tissues. It easily reacts with molecules such as amino acids, proteins, and DNA, thus resulting in cell damage. It was also believed to be an active initiator for peroxidation of lipids.[20] The inhibitory effect of NMCOS and NSCOS on hydroxyl radical was showed in Figure 2. With the increase of concentration, the scavenging effect on hydroxyl radical of NMCOS and NSCOS increased. IC_{50} of NMCOS was estimated as 0.24 mg/mL, however, IC_{50} of NSCOS could not be read. Although scavenging activity of NMCOS on hydroxyl radical was better compared to that of NSCOS on hydroxyl radical, scavenging activity of NMCOS on hydroxyl radical was weaker than that of COS which has a IC_{50} of 0.168 mg/mL.

It was reported that reducing power serves as a significant indicator of antioxidant activity for a compound. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radicals' chain by donating a hydrogen atom.[23] The reducing power of NMCOS and NSCOS was showed in Figure 3. The absorbances of NMCOS and NSCOS increased with their concentration increasing. At a concentration of 2.4 mg/mL, the absorbance of NMCOS and NSCOS was 0.46 and 0.41, respectively. It indicated that the reducing power of NMCOS was stronger than that of

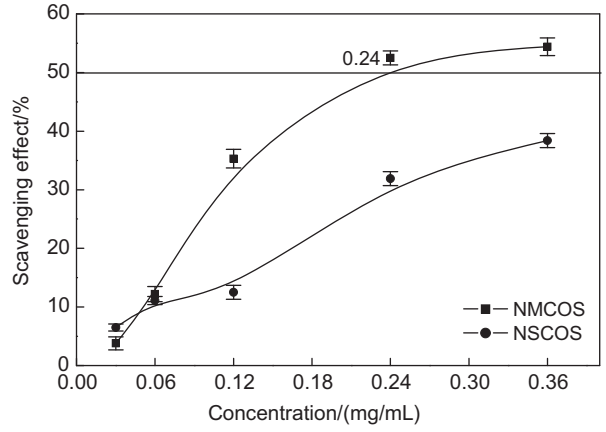

Figure 2. Scavenging effects of NMCOS and NSCOS on hydroxyl radical.

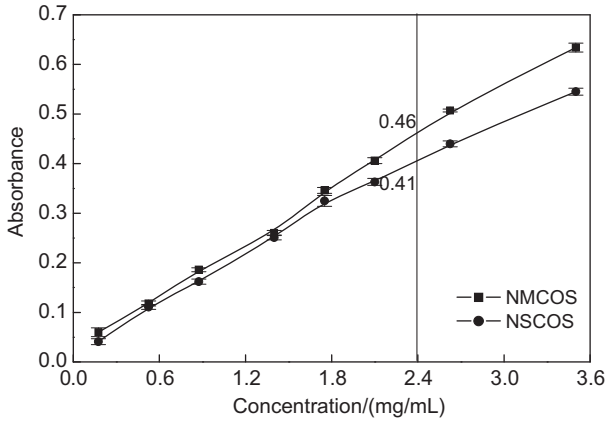

Figure 3. Reducing power of NMCOS and NSCOS.

NSCOS. However, COS had the best reducing power in this test system, its absorbance was 0.57 at the concentration of 2.4 mg/mL, which should mainly be owed to the fact no active amino groups had been substituted.

The antioxidant activity of chitosan oligosaccharide and its derivatives mainly attributed to the hydroxyl and amino groups. It indicated that the amount and the activity of hydroxyl and amino groups were the important factors associated with the antioxidant activity of chitosan oligosaccharide and its derivatives.[24] The properties of substituting groups may affect the antioxidant activity of chitosan and its derivatives in such way: firstly, the substitution will reduce the amount of active amino and hydroxyl groups in the polymer chains; secondly, the substitution may partly destroy the intermolecular and intramolecular hydrogen bonds. In present study, NMCOS and NSCOS have the same substituting degree, and the same content of hydroxyl and amino groups, but their scavenging effects on superoxide radical, hydroxyl radical and reducing power are different, which may be due to the activity of hydroxyl and amino groups. The NMCOS was considered as COS grafted by –COCH=CHCOO$^-$ group and the NSCOS was COS grafted by –$COCH_2CH_2COO^-$ group. Both –COCH=CHCOO$^-$ group and –$COCH_2CH_2COO^-$ group are electron-withdrawing groups, the electron-withdrawing effect of –COCH=CHCOO$^-$ group and the –$COCH_2CH_2COO^-$ group could destroy the intermolecular and intramolecular hydrogen bonds, and enhanced the activity of hydroxyl and amino groups. Furthermore, the electron-withdrawing effect of –COCH=CHCOO$^-$ group was stronger than that of –$COCH_2CH_2COO^-$ group, thus with the same substituting degree, the scavenging effects on superoxide radical, hydroxyl radical and reducing power of NMCOS were stronger than that of NSCOS.

The charge properties of substituting groups may affect the antioxidant activity of chitosan and its derivatives.[25,26] The property of substituting groups might affect the activity of amino and hydroxyl groups, therefore lead to the change of antioxidant activity. In present study, *N*-maleoyl chitosan oligosaccharide and *N*-succinyl chitosan oligosaccharide with the same substituting degree were synthesized. *N*-Maleoyl chitosan oligosaccharide showed the stronger antioxidant activity than *N*-succinyl chitosan oligosaccharide because of substituting group –COCH=CHCOO$^-$ has stronger electron-withdrawing effect than –$COCH_2CH_2COO^-$.

Acknowledgements

This work was supported by the Program of Shanghai Subject Chief Scientist (09XD1402000), Key Scientific Program of Shanghai Bio-Medical and Agricultural Science (08391911500) and Leading Academic Discipline Project of Shanghai Municipal Education Commission (J50704).

References and notes

1. Fernandes, J. C.; Eaton, P.; Nascimento, H.; Belo, L.; Rocha, S.; Vitorino, R.; Amado, F.; Gomes, J.; Silva, A. S.; Pintado, M. E.; Malcata, F. X. *Biomacromolecules* **2008**, *9*, 3346.
2. Xia, W. S.; Liu, P.; Zhang, J. L.; Chen, J. *Food Hydrocolloids* **2010**, *25*, 170.
3. Du, Y. Z.; Wang, L.; Dong, Y.; Yuan, H.; Hu, F. Q. *Carbohydr. Polym.* **2010**, *79*, 1034.
4. Kuroiwa, T.; Izuta, H.; Nabetani, H.; Nakajima, M.; Sato, S.; Mukataka, S.; Ichikawa, S. *Process Biochem.* **2009**, *44*, 283.
5. Fernandes, J. C.; Tavaria, F. K.; Soares, J. C.; Ramos, S. Ó.; Monteiro, M. J.; Pintado, M. E.; Malcata, F. X. *Food Microbiol.* **2008**, *25*, 922.
6. Eaton, P.; Fernandes, J. C.; Pereira, E.; Pintado, M. E.; Malcata, F. X. *Ultramicroscopy* **2008**, *108*, 1128.
7. Sun, T.; Yao, Q.; Zhou, D. X.; Mao, F. *Bioorg. Med. Chem. Lett.* **2008**, *21*, 5774.
8. Fernandes, J. C.; Eaton, P.; Nascimento, H.; Gião, M. S.; Ramos, Ó. S.; Belo, L.; Silva, A. S.; Pintado, M. E.; Malcata, F. X. *Carbohydr. Polym.* **2010**, *79*, 1101.
9. Vanichvattanadecha, C.; Supaphol, P.; Nagasawa, N.; Tamada, M.; Tokura, S.; Furuike, T.; Tamura, H.; Rujiravanit, R. *Polym. Degrad. Stab.* **2010**, *95*, 234.
10. Zhu, A. P.; Zhao, F.; Ma, T. *Acta Biomater.* **2009**, *5*, 2033.
11. Sui, W. P.; Wang, Y. H.; Dong, S. L.; Chen, Y. J. *Colloids Surf., B* **2008**, *316*, 171.
12. Wang, Z. Y.; Jiang, Z. J.; Hu, X. Q.; Shu, S. T.; Chen, F. *Chin. J. Appl. Chem.* **2002**, *19*, 1002.
13. Zhu, A. P.; Chen, T.; Yuan, L. H.; Wu, H.; Lu, P. *Carbohydr. Polym.* **2006**, *66*, 274.
14. Mu, Q.; Fang, Y. E. *Carbohydr. Polym.* **2008**, *72*, 308.
15. Hirano, S.; Sakaguchi, T.; Kuramitsu, K. *Carbohydr. Polym.* **1992**, *19*, 135.
16. Hirano, S.; Ohe, Y.; Ono, H. *Carbohydr. Res.* **1976**, *47*, 315.
17. Muzzarellia, R. A. A.; Tanfania, F.; Emanuellia, M.; Mariottia, S. *Carbohydr. Res.* **1982**, *107*, 199.
18. Sun, T.; Xie, W. M.; Xu, P. X. *Carbohydr. Polym.* **2004**, *58*, 379.
19. Yen, G. C.; Chen, H. Y. *J. Agric. Food. Chem.* **1995**, *43*, 27.
20. Halliwell, B.; Chirico, S. *Am. J. Clin. Nutr.* **1993**, *57*, 715.
21. Ji, X.; Zhong, Z. M.; Chen, X. L.; Xing, R. G.; Liu, S.; Wang, L. P. C. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4275.
22. Guo, Z. Y.; Xing, R. G.; Liu, S.; Yu, H. H.; Wang, P. B.; Li, C. P.; Li, P. C. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4600.
23. Duh, P. D.; Du, P. C.; Yen, G. C. *Food Chem. Toxicol.* **1999**, *37*, 1055.
24. Xie, W. M.; Xu, P. X.; Liu, Q. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1699.
25. Guo, Z. Y.; Liu, H. Y.; Chen, X. L.; Ji, X.; Li, P. C. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 6348.
26. Guo, Z. Y.; Xing, R. E.; Liu, S.; Zhong, Z. M.; Li, P. C. *Carbohydr. Polym.* **2008**, *73*, 173.