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Antioxidant activity of *N*-carboxymethyl chitosan oligosaccharides

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ABSTRACT

Three *N*-carboxymethyl chitosan oligosaccharides (*N*-CMCOSs) with different degrees of substitution (NA: 0.28, NB: 0.41, and NC: 0.54, respectively) were prepared by the control of the amount of glyoxylic acid in the etherification process of chitosan oligosaccharide (COS). Their antioxidant activities were evaluated by the scavenging of 1,1-diphenyl-2-picrylhrazyl radical (DPPH) radical, superoxide anion and determination of reducing power. With the increasing of substituting degree, the scavenging activity of *N*-CMCOSs against DPPH radical decreased and reducing power increased. As for superoxide anion scavenging, the order is NB > NC > NA. The difference may be related to the different radical scavenging mechanisms and donating effect of substituting carboxymethyl group.

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Chitosan is naturally occurring cationic polysaccharide consisting of D-glucosamine monomers linked through β-(1→4) glycosidic linkages. In order to improve the water solubility and enlarge the utilization of chitosan, chemical modification is widely applied to introduce a variety of functional groups in the polymer chains. Carboxymethyl chitosan is one of the most important kinds of chitosan derivatives. Based on the different substituting positions of carboxymethyl group, carboxymethyl chitosan could be divided into three kinds: *O*-carboxymethyl chitosan, *N*-carboxymethyl chitosan, and *N,O*-carboxymethyl chitosan.[1] The substituting positions and degrees of carboxymethyl group in the polymer chain will directly affect the properties of carboxymethyl chitosan.[2]

Recently antioxidant activity of chitosan and its derivatives has attracted the most attention due to their nontoxic nature and natural abundance. These researches showed that the antioxidant activity of chitosan and its derivatives mainly related to the content of active hydroxyl and amino groups in the polymer chains.[3–6] And with the decrease of molecular weights, the antioxidant activity of chitosan and its derivatives will be enhanced due to the partly destroying of intermolecular and intramolecular hydrogen bonds.[7–9]

Compared with chitosan, the antioxidant activity of chitosan oligosaccharide (COS) its derivatives will be much improved and may be more interesting because of less effect of hydrogen bonds.[10–12] Kim tried to reveal the relationship of antioxidant activities of COS derivatives and the physico-chemical properties.[13,14] However, there is little research on the relationship between the properties of carboxymethyl chitosan oligosaccharide and its substituting positions and degrees. In this paper, three *N*-carboxymethyl chitosan oligosaccharides (*N*-CMCOSs) with different substituting degrees of carboxymethyl were synthesized in order to investigate the effect of substituting groups on antioxidant activity and thus to deduce the antioxidant mechanisms of COS derivatives. Their antioxidant activity was investigated by scavenging DPPH radical, superoxide anion and determination of reducing power.

COS (4.0 g, 5000 Da, supplied by Zhejiang Jinke Biochemistry Co., Ltd) was dissolved in water (150.0 mL) with glyoxylic acid (0.2 g) and stirred for 2 h. The solution was adjusted to pH 9.0 by 10 wt% NaOH solution. Then, 10.0 mL $NaBH_4$ solution (10 wt%) was added and stirred for 2 h. The resultant solution was adjusted to pH 7.0, filtered, washed with alcohol repeatedly, then dried under vacuum at 60 °C to obtain *N*-carboxymethyl chitosan oligosaccharide A (NA). *N*-carboxymethyl chitosan oligosaccharide B and C (NB and NC) were prepared according to the similar procedure by using 0.4 and 0.8 g glyoxylic acid as etherifying agent, respectively. Yield changed gradually when the amount of glyoxylic acid changed. The yields of NA, NB, and NC were 53.75%, 57.5%, and 62.5%, respectively.

The degrees of substitution (DSs) of NA, NB, and NC were measured by pH titration according the following procedure.[2] *N*-CMCOSs samples (0.2 g) were dissolved in 20.0 mL HCl (0.1 M) and titrated with 0.1 M NaOH solution. According to the change of pH values, the DSs of NA, NB, and NC were calculated as 0.28, 0.41, and 0.54, respectively. The FTIR spectra of NA, NB, and NC all showed the characteristic absorption bands of COS.[15] The absorption of new peaks at around 1420 and 1600 cm^{-1} could be attributed to the stretch vibration absorption of $-CH_2COOH$ group and carboxyl group, respectively. Compared with the peaks of COS,

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the peaks of *N*-CMCOSs at 1589 cm^{-1} (*N*-H bend) decreased, which indicated that carboxymethyl groups had substituted the amino position of chitosan.

The DPPH scavenging activity of the samples was measured using the modified method of Yamaguchi et al.[16] 2.0 mL of methanolic solution of DPPH (0.1 mM) was incubated with varying concentrations of test samples (2.0 mL). The reaction mixture was shaken well and incubated for 30 min at 33 °C and the absorbance of the resulting solution was read at 517 nm against a blank. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following equation: Scavenging effect (%) = $(1 - A_{sample}/A_{control}) \times 100\%$.

The superoxide anion scavenging activity was determined using chemiluminescence technology.[17] The assay was carried out on a chemical luminometer (IFFM-D, Xi'an, China). The chemiluminescent reaction was processed in a Na_2CO_3–$NaHCO_3$ (pH 10.20, 0.5 M) buffer solution. 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 anion tested by superoxide dismutase, catalase and mannitol.

The reducing power of all samples was determined by the method of Yen and Chen.[18] Different concentrations of chitosan oligosaccharide derivative solutions (2.0 mL) were mixed with 2.5 mL sodium phosphate buffer (pH 6.6, 0.2 M) and 2.5 mL potassium fericyanide (1% W/V). The mixtures were incubated for 20 min at 50 °C, 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 significance (Originpro 6.1, $p < 0.05$).

Figure 1 depicted the DPPH radical scavenging effect of NA, NB, and NC. Scavenging activity of DPPH radical increased with the increasing of concentrations of NA, NB, and NC, this is, was concentration-dependent. Moreover, as shown in the figure, 50% inhibition concentrations (IC_{50}s) of NA, NB, and NC were 0.32, 0.42, and 0.71 mg/mL, respectively. In this system, the DPPH radical scavenging effect of COS was better than that of *N*-CMCOSs at all the tested concentrations, and the IC_{50} was 0.22. Considering the fact that COS may be regarded a special CMCOS with no carboxymethyl group substituted (DS is 0), the results showed that the scavenging effect on DPPH radical decreased with the increasing of the DSs. DPPH is one of the compounds that possessed a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical sacvengers.[15] Further it is well accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability.[19] Thus, scavenging of DPPH free radical was directly affected by the amount of attractable atoms in COS molecules.[13,14] Lower DSs resulted in more active amino groups and could donate more hydrogen to react with DPPH radical. Therefore, NA with the lowest DS had the strongest scavenging effect on DPPH radical.

Superoxide anion is formed in almost all aerobic cells and is a major agent in the mechanism of oxygen toxicity.[20] Superoxide anion is known to be very harmful to cellular components as a precursor of more reactive oxidative species, such as single oxygen and hydroxyl radicals. Compared with other oxygen radicals, superoxide anion has a longer lifetime, can move to an aim at a longer distance, and thus has more dangerous. Therefore, it is very important to study the scavenging of superoxide anion. Figure 2 showed the superoxide anion scavenging activity of *N*-CMCOSs at different concentrations. The scavenging effects of NA, NB, and NC increased with the increasing of concentrations. Their IC_{50}s were 3.48, 2.64, and 3.18 mg/mL, respectively. The results indicated that NB (its DS was 0.41) showed the strongest superoxide anion scavenging activity. As an electron-donating group, carboxymethyl group may enhance the electron cloud density of active hydroxyl and amino groups in the COS polymer chain. Thus, the electron-donating activity of *N*-CMCOSs increased and the scavenging effect on superoxide anion increased when DS increased from 0.28 to 0.41. Although the electron cloud density of active hydroxyl and amino groups increased while DS further improved to 0.54, the content of active amino groups decreased because of higher substitution degree and thus the electron-donating activity decreased, therefore the scavenging effect of NC decreased compared to NB.

Many researches suggested the scavenging mechanism of chitosan is based on that superoxide anion can react with active hydrogen atoms in chitosan to form a most stable macromolecular radical.[7–9,17] The antioxidant activity of Schiff bases of chitosan and carboxymethyl chitosan reduced because of the amino and hydroxyl were substituted.[21] Similarly, in this antioxidant evaluation system COS had better superoxide anion scavenging activity compared with *N*-CMCOSs (the IC_{50} was 1.92 mg/ml) because of no active amino groups substituted. Superoxide anion is a zwitterionic radical. It could react with free hydroxyl and amino groups in COS. Then superoxide anion was eliminated by this reaction.

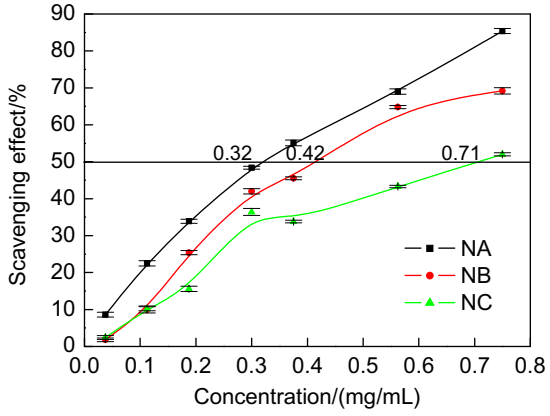

Figure 1. Scavenging effects of *N*-CMCOSs on DPPH radical.

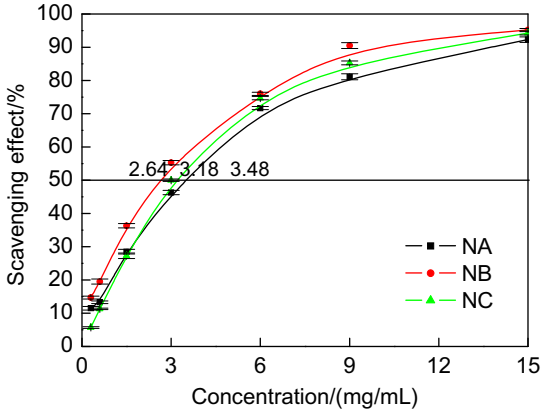

Figure 2. Scavenging effects of *N*-CMCOSs on superoxide anion.

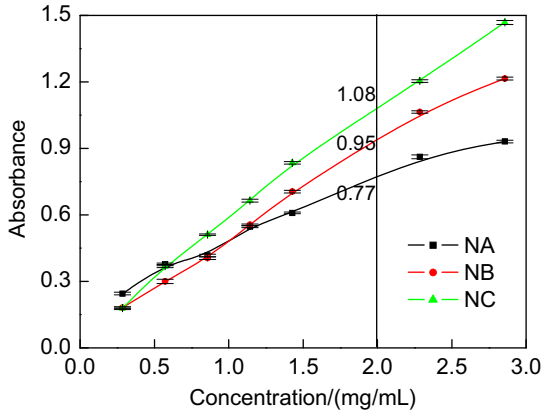

Figure 3. Reducing power of *N*-CMCOSs.

Reducing power assay has also been used to evaluate the ability of natural antioxidants to donate electrons.[22] The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Figure 3 depicted the reducing power of NA, NB, and NC. The absorbances of NA, NB, and NC increased with the increasing of their concentrations. At a concentration of 2.0 mg/mL, the absorbance of NA, NB, and NC were 0.77, 0.95, and 1.08, respectively. The data showed that the reducing power increased with the increasing of DS, which indicated that carboxymethyl group polymerized on COS increased the reducing power of *N*-CMCOSs obviously. However, COS had the best reducing power in this test system, its absorbance was 1.29 at the concentration of 2.0 mg/mL, which should mainly be owed to the fact no active amino groups had been substituted. A direct correlation between antioxidant activities and reducing power of certain plant extracts has been reported. The reducing power properties are generally associated with the presence of reductions, which have been shown to exert antioxidant action by breaking the free radicals' chain by donating a hydrogen atom.[23] This result may be related to the fact that the introduction of electron-donating carboxymethyl group enhanced the electron cloud density of active hydroxyl and amino groups, thus the electron-donating activity increased and the reducing power improved.

The charge properties of substituting groups may affect the antioxidant activity of chitosan and its derivatives.[24,25] However, the effect of properties of substituting groups cannot be obvious because of the facts: 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. Compared with chitosan, COS has soft hydrogen bonds, which is helpful to investigate the effect of substituting groups on antioxidant activity of COS derivatives. In present study, three *N*-carboxymethyl chitosan oligosaccharides with various substituting degrees were prepared. They had diverse antioxidant activities in the antioxidant systems. The scavenging effect on DPPH radical decreased with the increasing of DS, while the reducing power increased with the increasing of DS. In addition, the order of their inhibiting efficacy on superoxide anion was: NB > NC > NA. That may be related with the different radical scavenging mechanisms and donating effect of substituting carboxymethyl group.

Acknowledgments

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