Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 1699–1701

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Antioxidant Activity of Water-Soluble Chitosan Derivatives

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Received 9 January 2001; accepted 16 April 2001

Abstract—Water-soluble chitosan derivatives were prepared by graft copolymerization of maleic acid sodium onto hydroxypropyl chitosan and carboxymethyl chitosan sodium. Their scavenging activities against hydroxyl radical ·OH were investigated by chemiluminescence technique. They exhibit IC_{50} values ranging from 246 to 498 μg/mL, which should be attributed to their different contents of hydroxyl and amino groups and different substituting groups.

Chitosan is a cationic polysaccharide made from alkaline *N*-deacetylation of chitin. It has attracted much attention as a biomedical material, owing to its unique biological activities such as antitumor, antiulcer, immunostimulatory, antibacterial and so on. The applications of chitosan are limited because of the insolubility at neutral or high pH region. So it is important to improve the soluble property of chitosan. In this paper, maleic acid sodium (MAS) was grafted onto hydroxypropyl chitosan (HPCT) and carboxymethyl chitosan sodium (CMCTS) to prepare water-soluble chitosan derivatives: HPCT-g-MAS and CMCTS-g-MAS, respectively.

Recently, the antioxidant activity of chitosan and its derivatives has attracted the most attention.[1–5] Among various reactive oxygen species, the chemical activity of hydroxyl radical ·OH is the strongest, which can easily react with biomolecules such as amino acids, proteins, and DNA.[6] Here, the antioxidant activity of water-soluble chitosan derivatives was estimated as hydroxyl radical scavengers by chemiluminescence (CL) technique.

Chitosan (3.0 g, degree of deacetylation: 97%, $Mv = 8.8 \times 10^5$, supplied by Zhejiang Yuhuan Biochemistry Co. Ltd.) was added into 15.0 g 50 wt% NaOH solution and put into a refrigerator at −18 °C for alkalization. Alkali chitosan and isopropyl alcohol (30.0 mL) were added into a 100 mL reactor and stirred for 1 h at 40 °C. Then, 30.0 mL propylene epoxide was added, and refluxed for 2 h at 60 °C. The resultant solution was adjusted to pH 7.0, filtered, repeatedly washed with acetone and 95% (v/v) alcohol, then dried under vacuum at 60 °C for 48 h to obtain HPCT. Elemental analysis results were C, 43.23 (43.11); N, 5.74 (5.72); and H, 6.98 (6.94). The IR spectrum showed not only the characteristic absorption bands of chitosan but also a new peak at 2970 cm^{-1}, indicating incorporation of the hydroxypropyl moiety. In the ^1H NMR spectroscopy, the protons of the hydroxypropyl moiety successively absorb at δ 3.10, 5.05, and 3.80 ppm.[7]

CMCTS was prepared according to a similar procedure, except that chloroacetic acid replaced propylene epoxide and should be added dropwise. Elemental analysis results were C, 39.13 (38.93); N, 5.76 (5.67); and H, 5.83 (5.43). CMCTS was confirmed by absorption bands (IR) in the 1410 [γ_{sym} (CO_2)] and 1596 cm^{-1} [γ_{as} (CO_2)], and proton absorption (^1H NMR) at δ 3.39 ppm.

The grafted copolymers were prepared as follows. 0.20 g HPCT or CMCTS and a predetermined amount of MAS were added into a 100 mL reactor, and stirred for 30 min under nitrogen atmosphere with heating to 70 °C. 0.1 mmol ammonium persulfate was dissolved in 10 mL H_2O, then slowly added into the reactor to initiate the graft copolymerization. Reaction products were precipitated in acetone, filtered, repeatedly washed with acetone and dried at 60 °C under vacuum. Homopolymers were extracted in a Soxhlet apparatus by refluxing in alcohol for 24 h and dried at 60 °C under vacuum for 48 h. Graft percentage was calculated as: $G/\% = [(W_2 - W_1)/W_1] \times 100$. Where W_1 represents weight of CMCTS or HPCT, W_2 weight of grafted copolymer. The IR spectra of grafted copolymers all showed peaks at around 1700 cm^{-1} and characteristic broad bands of carboxylate at 1560–1520 cm^{-1}.

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0960-894X/01/$ - see front matter
PII: S0960-894X(01)00285-2

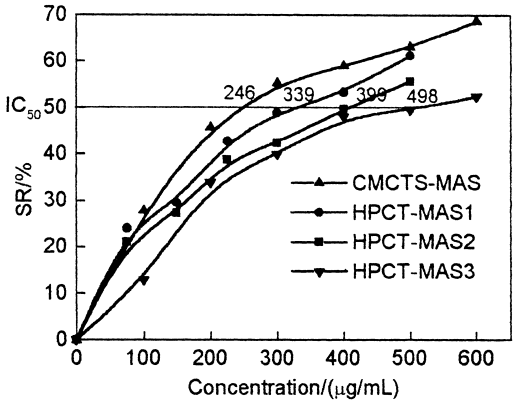

Figure 1. Plot of clearance versus concentration of chitosan derivatives.

Table 1. Graft polymerization of MAS onto CMCTS or HPCT at 70 °C[a]

Copolymer	Concentration (mol/L)	G (%)	IC_{50} (μg/mL)
CMCTS-g-MAS	0.6	586	246
HPCT-g-MAS1	0.6	560	339
HPCT-g-MAS2	1.2	1633	399
HPCT-g-MAS3	1.8	1720	498

[a]Reaction conditions: CMCTS: 0.20 g; HPCT: 0.20 g; APS: 0.1 mmol; 2 h.

•OH was produced by a copper-catalyzed Haber–Weiss reaction, and zymosan was used as a CL amplifier.[8] Chitosan derivatives were dissolved in the buffer of NaH_2PO_4/Na_2HPO_4 (pH 7.8). To the flat glass tube, ascorbic acid (Vc), zymosan, samples, and H_2O_2 were added in their given order (samples were replaced by the corresponding buffer solution in the control group) and their final concentrations were as follows: $CuSO_4$ (0.4 mmol/L), Vc (0.2 mmol/L), zysoman (2.5 mg/mL), H_2O_2 (20 mmol/L). The chemiluminigenic emission from the reaction mixtures was immediately counted at intervals of 15 s for 100 times. The amount of hydroxyl radical was represented by the peak value in the CL–t curve. Thus the scavenging rate (SR) of test sample was calculated as: $SR/\% = [(CL_0–CL_1)/CL_0]\times 100$ where CL_0, CL_1 represent peak values in the CL–t curves of the control group and test group, respectively. Every data point was obtained from three parallel determinations. The tolerance was no more than 3%. The free radical produced in this system was proved to be hydroxyl radical •OH tested by superoxide dismutase (SOD), catalase, and mannitol. Thiourea and benzoic acid were used as a control.

The scavenging effects of chitosan derivatives on •OH are shown in Figure 1. Four compounds have obvious scavenging activity. The scavenging rate increases with concentration. The scavenging mechanism may be related to the fact that •OH can react with active hydrogen atoms in chitosan to form a most stable macromolecule radical.[9] The scavenging activities of chitosan derivatives against •OH may be derived from some or all of the following:

i. The hydroxyl groups in the polysaccharide unit can react with •OH by the typical H-abstraction reaction.
ii. •OH can react with the residual free amino groups NH_2 to form stable macromolecule radicals.
iii. The NH_2 groups can form ammonium groups NH_3^+ by absorbing hydrion from the solution, then reacting with •OH through addition reaction.

The IC_{50} of CMCTS-g-MAS, HPCT-g-MAS1, 2, 3 are, respectively, 246, 339, 399, and 498 μg/mL. Their different scavenging effects on •OH should be attributed to their different structures. As shown in Table 1, the IC_{50} is high when the grafting percentage is high, which indicates that the copolymer with low content of chitosan has low scavenging ability. At high grafting percentage, the copolymer has relatively low content of hydroxyl and amino groups, and thus low scavenging ability.

CMCTS-g-MAS has high scavenging effect on •OH, which should also be attributed to the reactivity of OH and NH_2 groups. It is well known that the free radicals' scavenging activities are closely related to bond dissociation energy of O–H or N–H and the stability of the formed radicals. Chitosan has strong intramolecular and intermolecular hydrogen bonds. The OH and NH_2 groups are difficult to dissociate and react with •OH. So chitosan has almost no antioxidant activity, which was proved by Alexandrova et al.[2] Compared with HPCT, CMCTS has a substituting carboxylic group which is a stronger electron-withstanding group than hydroxypropyl group. The electron-withstanding group improves the energy level of the highest occupied molecular orbital (HOMO) and declines the dissociation energy of O–H or N–H simultaneously.[10] Therefore, CMCTS-g-MAS and HPCT-g-MAS1 have similar grafting percentages, and similar content of OH and NH_2, but different scavenging effects on •OH.

In this system, the IC_{50} of thiourea, mannitol, and benzoic acid are 180, 2500, and 3500 μg/mL, respectively. Chitosan derivatives have similar hydroxyl radical scavenging ability as thiourea, but better than mannitol and benzoic acid. The antioxidant activity of chitosan derivatives will be helpful to expand their applications in biomedicine.

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