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Preparation of low-molecular-weight and high-sulfate-content chitosans under microwave radiation and their potential antioxidant activity in vitro

Ronge Xing,[a,b] Song Liu,[a,b] Huahua Yu,[a,b] Quanbin Zhang,[a] Zhien Li[a] and Pengcheng Li[a,*]

[a]*Institute of Oceanology, The Chinese Academy of Sciences, Qingdao 266071, China*
[b]*Graduate School of the Chinese Academy of Sciences, Beijing 100039, China*

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Abstract—In the present paper microwave radiation has been used to introduce *N*-sulfo and *O*-sulfo groups into chitosan with a high degree of substitution and low-molecular weight. The sulfation of chitosan was performed in microwave ovens. It was found that microwave heating is a convenient way to obtain a wide range of products of different degrees of substitution and molecular weight only by changing reaction time or/and radiation power. Moreover, microwave radiation accelerated the degradation of sulfated chitosan, and the molecular weight of sulfated chitosan was considerably lower than that obtained by traditional heating. There are no differences in the chemical structure of sulfated chitosan obtained by microwave and by conventional technology. FTIR and ^{13}C NMR spectral analyses demonstrated that a significantly shorter time is required to obtain a satisfactory degree of substitution and molecular weight by microwave radiation than by conventional technology. In this present paper, we also determined antioxidant activity of low-molecular-weight and high-sulfate-content chitosans (LCTS). The results showed LCTS could scavenge superoxide and hydroxyl radical. Its IC_{50} is 0.025 and 1.32 mg/mL, respectively. It is a potential antioxidant in vitro.

Keywords: Chitosans, low-molecular-weight and high-sulfate-content; Microwave radiation; Traditional heating; Antioxidant activity; Superoxide radical; Hydroxyl radical

1. Introduction

In general the influence of molecular weight and/or substitution degree of sulfated polysaccharides on their biological activity is considered in the majority of works involving the anticoagulant or antiviral properties of these substances.[1,2] Sulfated chitosans have obvious anticoagulant activity because of their structural similarity to heparin. So, their preparation has also attracted the most attention. Previous studies have focused mainly on high-molecular-weight sulfated chitosan and preparation by traditional heating preparation. Xu and Xiao reported that chitosan was sulfated in sulfuric acid (95%, 90%, 80%) at about −10–0 °C for 3 h, and the molecular weight of the resulting product was 2.51×10^4;[3] Wu et al. and Li et al. prepared chitosan sulfates with chlorosulfonic acid at 70 °C for 4 h;[4,5] Vongchan et al. dropped solvated chitosan to the sulfating complex (4.5 mL of $HClSO_3$ in cold DMF) and stirred at RT for 5 h.[6] However, few people have studied low-molecular-weight sulfated chitosan, especially, none on microwave radiation preparation.

Abbreviations: Low-molecular-weight and high-sulfate-content chitosans (LCTS); reactive oxygen species (ROS); *N*,*N*-dimethylformamide (DMF); Nitro Blue tetrazolium (NBT); phenazine methosulfate (PMS); hydrogen peroxide (H_2O_2); thiobarbituric acid (TBA); ethylenediaminetetraacetic acid (EDTA); nicotinamide adenine dinucleotide-reduced (NADH); trichloroacetic acid (TCA); deoxyribose (DR).

* Corresponding author. Tel.: +86 532 2898707; fax: +86 532 2968951; e-mail: pcli@ms.qdio.ac.cn

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Microwave irradiation using commercial domestic microwave ovens has received increasing interest in organic synthesis due to remarkable enhancements of the rates of some organic reactions comparing to conventional reaction.[7,8] Moreover, microwave radiation (2450 MHz) does not activate specific bonds in molecules, and consequently this form of heating will not lead to any kinetic differences compared to other forms of heating.[9] Significant kinetic effects for some Diels–Alder reactions have been also reported,[10] but not for esterification. In this paper LCTS was prepared under microwave radiation, and their structural features were elucidated by FTIR and ^{13}C NMR spectroscopy.

Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various degenerative diseases as well as in the normal process of aging.[11] Reactive oxygen species (ROS) in the forms of superoxide anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), and hydrogen peroxide (H_2O_2) are generated by the normal metabolic process or from exogenous factors and agents, and they can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. These ROSs are capable of damaging a wide range of essential biomolecules.[12] Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. They exert their effects by scavenging ROSs, activating a battery of detoxifying proteins, or preventing the generation of ROSs.[13] In recent years, there has been increasing interest in finding natural antioxidants, since they can protect the human body from free radicals and retard the progress of many chronic diseases.[14] For example, Yin et al. reported that low-molecular-weight chitosan could scavenge superoxide radicals, and its scavenging activity was 80.3% at 0.5 mg/mL.[15] Esumi et al. showed that gold–chitosan nanocomposites have an ability to depress the activity of hydroxyl radicals.[16] In this paper, LCTS was studied as a new antioxidant. We found that it could scavenge superoxide/hydroxyl radicals. It was a potential antioxidant.

2. Experimental

2.1. Materials and apparatus

Chitosan derived from shrimp shells (Qingdao Baicheng Biochem. Corp., China) was used without further purification. Nitro Blue tetrazolium (NBT), phenazine methosulfate (PMS), hydrogen peroxide (H_2O_2), thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), ferrozine, nicotinamide adenine dinucleotide-reduced (NADH), trichloroacetic acid (TCA), and deoxyribose (DR) were purchased from Sigma Chemical Co. All other chemicals and reagents, unless otherwise specified, were not purified, dried, or pretreated. Dialysis membranes were bought from Sigma Chemical Co., molecular weight cut off at 3600 Da. A domestic microwave oven of 2.45 MHz (Tianjin Yuejin Electric and Electrical Appliance Limited Liability Company, WD850 (MG-5588SDTW)) was used.

2.2. Analytical methods

The degree of deacetylation of chitosan was 87% by potentiometry, and the viscosity average-molecular weight was 7.6×10^5. FTIR spectra were measured by a Nicolet Magna-Avatar 360 with KBr disks; ^{13}C NMR spectra were recorded on a Bruker Apx 500 (500 MHz) NMR spectrometer in D_2O solvent. Sulfate content % was measured in a SC-132 sulfur meter (LECO). The average viscometric molecular weight of sulfated chitosan was estimated from the intrinsic viscosity determined in the solvent 0.1 M CH_3COOH/0.2 M NaCl using the Mark–Houwink parameters $\alpha = 0.96$, $K_\eta = 1.424$ at 25 °C when the intrinsic viscosity was expressed in mL g^{-1}.[17]

2.3. Preparation of sulfating reagent

$HClSO_3$ (5.0 mL) was added dropwise with stirring to 30 mL of *N*,*N*-dimethylformamide (DMF) previously cooled at 0–4 °C. The reaction mixture was stirred without cooling until the solution reached room temperature. Abbreviation of the sulfating reagent is: DMF·SO_3.

2.4. Preparation of chitosan sulfates

DMF·SO_3 reagent (50 mL) was added to a 300-mL Erlenmeyer flask containing 50 mL of chitosan solution in a mixture of DMF–formic acid with swirling to get gelatinous chitosan. The Erlenmeyer flask containing the mixture of reactant was placed on the center of the turntable of the microwave oven. To control the reaction temperature to ~100 °C, another 50-mL Erlenmeyer flask containing a higher boiling solvent was also placed on the turntable in the microwave oven. Different irradiation powers and radiation times were set. After irradiation ceased, the reaction liquid was immediately poured into 90% EtOH (300 mL), giving a white precipitate. The mixture of products was filtered through a Buchner funnel under reduced pressure. The precipitate was washed with EtOH, then redissolved in distilled water, and the pH was adjusted to pH 7–8 with 2 M NaOH. The solution was dialyzed against distilled water for 48 h using a 3600 Da MW cutoff dialysis membrane. The product was then concentrated and lyophilized to give chitosan sulfate (2 g chitosan gave 1.8–3.1 g chitosan sulfated according to different conditions, including radiation power, radiation time, etc.) with a sulfur content of 8–15%, which corresponds to a degree of sulfation of 1.12–2.10 per glucosamine unit.

2.5. Superoxide radical-scavenging assay

The superoxide scavenging ability of the extract was assessed by the method of Nishikimi et al.[18] The reaction mixture, containing LCTS (0.005–0.4 mg/mL), PMS (30 μM), NADH (338 μM), and NBT (72 μM) in phosphate buffer (0.1 M pH 7.4), was incubated at room temperature for 5 min, and the absorbance was read at 560 nm against a blank. The capability of scavenging the superoxide radical was calculated using the following equation:

$$\text{Scavenging effect } (\%) = \left(1 - \frac{A_{\text{sample 560 nm}}}{A_{\text{control 560 nm}}}\right) \times 100$$

2.6. Hydroxyl radical assay

The reaction mixture containing LCTS (0.1–3.2 mg/mL), was incubated with deoxyribose (3.75 mM), H_2O_2 (1 mM), $FeCl_3$ (100 μM), EDTA (100 μM), and ascorbic acid (100 μM) in potassium phosphate buffer (20 mM, pH 7.4) for 60 min at 37 °C.[13] The reaction was terminated by adding 1 mL of TBA (1% w/v) and 1 mL of TCA (2% w/v) and then heating the tubes in a boiling water bath for 15 min. The contents were cooled, and the absorbance of the mixture was measured at 535 nm against reagent blank. Decreased absorbance of the reaction mixture indicated decreased oxidation of deoxyribose.

3. Results and discussion

3.1. Effect of microwave radiation and time on LCTS

Table 1 shows the results of sulfated chitosan under different periods of time in a microwave field. Entry 9 showed that the molecular weight of sulfated chitosan was 1.25×10^5 in 1 h by means of conventional heating. Compared with this result, the result of other entries showed that microwave radiation accelerated the degradation of sulfated chitosan, and the molecular weight of sulfated chitosan was considerably lower than 1.25×10^5. Moreover, as shown in Table 1 the molecular weight changed abruptly at the time of 80 s. Namely the molecular weight at 80 s decreased 10^4 Da compared to that at 70 s. However, the molecular weight slowly changed over at 80 s. From 90–100 s the molecular weight decreased by only 2000 Da. Compared with conventional heating, microwave dielectric heating is an efficient procedure that shortens the reaction time. Microwave heat involves a direct interaction with certain classes of absorbing molecules. This direct absorption can lead to localized introduction of energy to a region from the remote microwave source and raises the solution temperature. It is well established that the superheating effects caused by microwave radiation can lead to temperatures 10–30 °C in excess of the conventional boiling point of the solvent. Also it is possible that localized superheating effects could lead to a small but significant increase in the reaction rate and the selectivity.[19] The chitosan sulfation and degradation reactions can thus be completed in a short time under microwave radiation.

Furthermore, as shown in Table 1, it is possible to obtain a wide range of products of different degrees of substitution and molecular weights using the same chitosan–chemicals blend by changing only the irradiation time or power conditions. An increase in reaction time or radiation power caused a decrease in all the examined parameters, including the degree of sulfur substitution, yield, and molecular weight. However, the color of the product was deepened under prolonged reaction times or with stronger radiation power. This indicates that in a prolonged reaction time or more powerful radiation power, subsequent degradation and slight desulfurization reactions can take place. All these results showed that the chitosan sulfation reaction depends on both reaction time and radiation power.

Otherwise, entry 10 showed a molecular weight of degradable chitosan under microwave radiation for 10 min. Its molecular weight was between those entries of 1–8 and entry 9. The results showed that microwave

Table 1. Chitosan sulfates obtained under microwave radiation at different power settings and reaction time[a]

Entry	Time (s)	Power (W)	Color of product	Solubility	Yield (%)	Molecular weight ($\times 10^3$)	Sulfur content (%)
1	70	480	Pale yellow	Easy soluble	153.0	26.6	13.7
2	70	640	Yellow	Easy soluble	148.9	21.6	14.7
3	70	800	Pale yellow	Easy soluble	97.9	10.9	11.7
4	80	480	Pale yellow	Easy soluble	143.7	16.8	14.9
5	90	480	Pale yellow	Easy soluble	128.9	11.4	14.5
6	100	480	Pale yellow	Easy soluble	100.8	9.09	13.5
7	110	480	Yellow	Easy soluble	84.3	7.69	13.4
8	120	480	Deep yellow	Easy soluble	75.3	4.41	12.7
9	1 h[b]	45 °C	Pale yellow	Soluble	177.5	125	14.5
10[c]	10 min	800	Yellow	Not soluble	57.0	34.6	0

[a] Reaction was carried out with 2 g of chitosan, 50 mL of sulfating reagent (DMF·SO_3).
[b] With conventional heating to replace microwave radiation to maintain the reaction volume.
[c] Chitosan degraded under microwave radiation: reaction was carried out with 2 g of chitosan, 80 mL of 0.2 M HCl.

radiation could accelerate the degradation of chitosan in spite of a considerably low concentration of HCl.

The structure of sulfated chitosan (prepared in this study) was further investigated by means of FTIR and ^{13}C NMR spectroscopy (Figs. 1 and 2). In the FTIR spectrum (as shown in Fig. 1), characteristic absorptions at 1222 and 806 cm^{-1}, due to sulfo groups, were assigned to S=O and C–O–S bond stretchings, respectively. The peak at 940 cm^{-1}, due to the pyranose units in the polysaccharide, proved that the cyclic pyranosyl rings were not destroyed by microwave radiation.[6,20] Moreover, the position of sulfur substitution could be shown by the ^{13}C NMR spectrum. In the ^{13}C NMR spectrum (as shown in Fig. 2), the signals of the parent chitosan at 65 and 52 ppm were assigned to the groups of the C-6 hydroxyl group and the C-2 amido group, respectively. After chitosan sulfation, the two peaks of chitosan were shifted to low field at 69 and 58 ppm, respectively. The existence of strong sulfated signals and the disappearance of unmodified (parent) signals indicated that the C-6 hydroxyl group and C-2 amido group were both completely sulfated. Two signals at 73 and 76 ppm belong to C-3 of the residue without sulfate and C-3 of the residue with sulfate, respectively. This shows that the C-3 hydroxyl groups were partly sulfated.[21,20] As expected, the C-3 hydroxyl groups were incompletely substituted because of steric hindrance limitations. Above-mentioned result showed that the sulfo groups had been successfully introduced to chitosan.

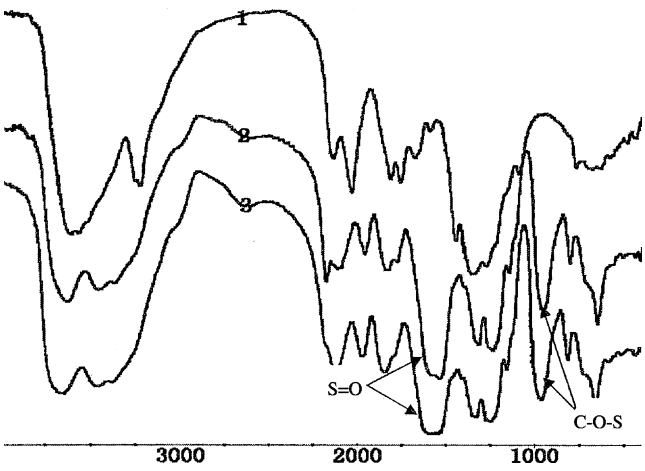

Figure 1. FTIR of sulfated chitosan, 1: chitosan; 2: sulfated chitosan under traditional heating; 3: sulfated chitosan under microwave radiation (800 W).

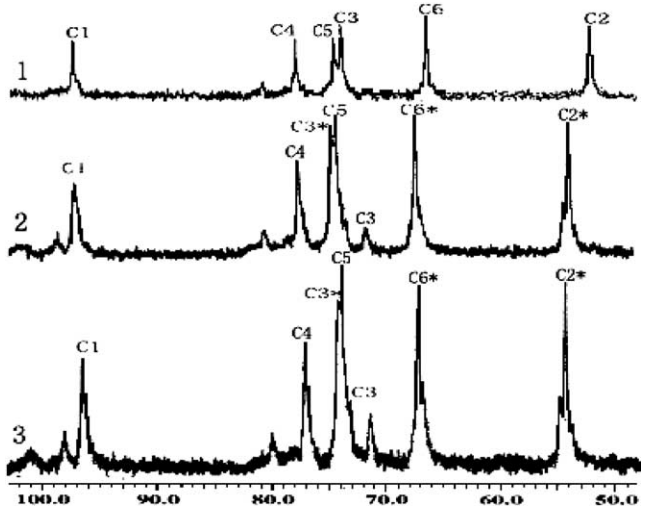

Figure 2. ^{13}C NMR of low-molecular-weight sulfated chitosan, 1: chitosan; 2: sulfated chitosan under traditional heating; 3: sulfated chitosan under microwave radiation (800 W). c* = signals of modified groups.

3.2. Antioxidant activity determination for LCTS

In this paper, the product of 480 W, 90 s irradiation was chosen for the antioxidant assay for the sake of its molecular weight, yield, and sulfur content. Figure 3 showed that the inhibitory effect of LCTS on superoxide radicals was marked and concentration related. A significant scavenging effect (24.13–92.51%) of superoxide radicals was evident at all tested concentration of LCTS (0.005–0.4 mg/mL). Moreover, as shown in Figure 3, the scavenging activity of superoxide radicals had reached 90% at 0.025 mg/mL. In comparison, low-molecular-weight chitosan and parent chitosan show scavenging activities for superoxide radicals of 80.3% and 13% at 0.5 mg/mL, respectively.[15] Although superoxide is a relatively weak oxidant, it decomposes to form a stronger reactive oxidative species, such as singlet oxygen and hydroxyl radicals, which initiate peroxidation of lipids.[22] In the present study, LCTS effectively scavenged superoxide in a concentration-dependant manner. Further, superoxides are also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating

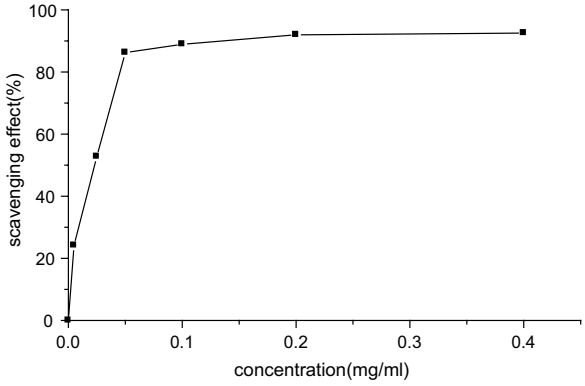

Figure 3. Scavenging effect of LCTS on superoxide radical. Values were means ± SD of three determinations. LCTS (480 W, 90 s) was chosen for antioxidant assays.

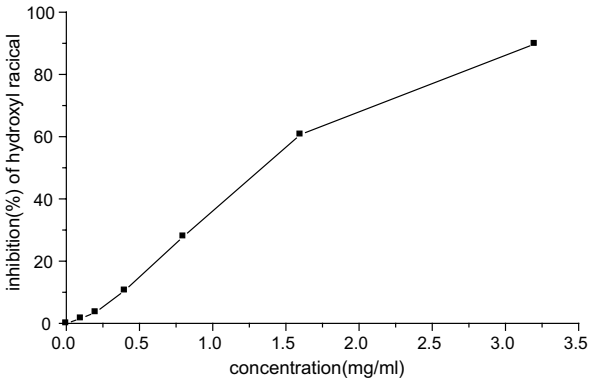

Figure 4. Inhibitory effect of LCTS on deoxyribose oxidative damage. Values were means ± SD of three determinations. LCTS (480 W, 90 s) was chosen for antioxidant assays.

precursors of hydroxyl radicals.[23] These results showed that LCTS has strong scavenging activity for the superoxide radical and clearly suggested that the antioxidant activity of LCTS was also related to its ability to scavenge superoxide radical.

The effect of LCTS on oxidative damage, induced by Fe^{3+}/H_2O_2 on deoxyribose, as measured by the thiobarbituric acid method, is plotted in Figure 4. Nearly 89.82% inhibition was observed at the highest concentration (3.2 mg/mL). Hydroxyl radical-scavenging activity of LCTS was also obtained in the deoxyribose system. In this system, LCTS exhibited a stronger concentration-dependent inhibition of deoxyribose oxidation. Earlier, numerous workers[13] have employed this system to assess the biological activity of various natural plant-derived biomolecules. Smith et al.[24] earlier reported that molecules that can inhibit deoxyribose degradation are those that can chelate iron ions and render them inactive or poorly active in a Fenton reaction. In the present study, it is likely that the chelating effect of LCTS on metal ions may be responsible for the inhibition of deoxyribose oxidation. However, the mechanism of LCTS on hydroxyl radicals needs to be further researched.

4. Conclusions

Microwave heating is a convenient way to obtain a low-molecular-weight and high-sulfur-content chitosan sulfate products. Several features of microwave heating are noted as follows:

1. Microwave radiation does not affect the kind of chemical groups substituted into chitosan as compared to the conventional sulfation process.
2. Microwave radiation does not affect the pyranose rings of polysaccharides as compared to products of traditional technology.
3. Compared with conventional heating, microwave dielectric heating is an efficient procedure that produced low-molecular-weight chitosan sulfate in a shorter time.
4. LCTS can scavenge superoxide/hydroxyl radicals. Its IC_{50} was 0.025 and 1.32 mg/mL, respectively. It is thus considered a potential antioxidant.

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