Free Radical Scavenging Activity of Cellulase-Treated Chitosan

Tao Feng,[1] Jian Gong,[2] Yumin Du,[3] Zili Huang[1]

[1]*Department of Environmental Engineering, College of Resources and Environmental Engineering, Wuhan University of Science and Technology, Wuhan 430081, China*
[2]*Department of Land Management, College of Resources, Chinese University of Geosciences, Wuhan 430074, China*
[3]*Department of Environmental Science, Wuhan University, Wuhan 430079, China*

Received 26 February 2007; accepted 21 July 2008
DOI 10.1002/app.29115
Published online 9 October 2008 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Partially *N*-acetylated chitosan was hydrolyzed by the cheap, commercially available, and efficient cellulase. The products, with different molecular weight, were comparatively investigated by GPC, FT-IR, XRD, and NMR. The results show that the decrease of molecular weight led to transformation of crystal structure and increase of water-solubility, but the chemical structures of residues were not modified. Superoxide anion radical and hydroxyl radical quenching assay were used for the evaluation of free radical scavenging activity of cellulase-treated chitosan *in vitro*. Low molecular weight chitosan (LMWC3, M_w 1.7 × 10^3) exhibited high scavenging activity against free radical. It scavenged 79.3% superoxide radical at 0.1 mg mL^{-1}. At 2.0 mg mL^{-1}, scavenging percentage of initial chitiosan, LMWC1 (M_w 27.3 × 10^3), LMWC2 (M_w 5.9 × 10^3), and LMWC3 (M_w 1.7 × 10^3) against hydroxyl radical was 14.3%, 33.1%, 47.4%, and 65.9%, respectively. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 111: 545–550, 2009

Key words: chitosan; molecular weight; cellulase; free radical; scavenging

INTRODUCTION

Chitin, a linear polysaccharide composed of 1,4-linked 2-acetamido-2-deoxy-β-D-glucose units, is one of the most abundant, easily obtained, and renewable natural polymers. And chitosan is the *N*-deacetylated derivative of chitin. The amino polysaccharide has received much attention as a functional biopolymer for many diverse applications in pharmaceutics[1] and food, including bioconversion for the production of value-added food products, preservation of food from microbial deterioration, purification of water, and clarification and deacidification of fruit juices.[2] These functions have been revealed to be dependent upon not only their chemical structure but also the molecular size. With the emergence of the potential biomedical and food applications for lower molecular weight chitosan (LMWC), the development of viable processes for the degradation of chitosan is attracting growing interest.[3] Cellulase, a cheap, commercially available enzyme, which is widely produced in nature by many bacteria, fungi and plants, is one of the nonspecific enzymes that can hydrolyze chitosan efficiently.[4,5]

Formation of free radical such as superoxide anion radical and hydroxyl radical is an unavoidable consequence in aerobic organisms during respiration. These radicals are very unstable and react rapidly with biomolecules including lipids, proteins, amines, lipoproteins, carbohydrates, and DNA in the body, leading to cell or tissue injury.[2] Various exogenous antioxidants play an important role in the elimination of free radicals and protect the cells against toxic affects of free radicals. The most commonly used antioxidants at present are butylated hydroxyanisole, butylated hydroxytoluene, *t*-butylhydroquinone, and propyl gallate.[6] However, the use of synthetic antioxidants is under strict regulation because of the potential health hazards caused by such compounds. Therefore, during past few decades, interest had been developed to search for effective natural antioxidants from different sources for use in foods or medicinal materials to replace synthetic antioxidants. Recently, the free radical scavenging activity of chitosan and its derivatives has attracted the most attention. The results of these studies have shown that they exert stronge activities and their effects are also similar to those of phenolic antioxidants. The radical scavenging activity of chitosan increased with a dose-dependent manner, and it was dependent on their degree of deacetylation (DD) values and molecular weights.[7,8]

Correspondence to: T. Feng (fengtaowhu@163.com).
Contract grant sponsor: Hubei Provincial Department of Education; contract grant number: D20081106.

© 2008 Wiley Periodicals, Inc.

Considering multifunction and low toxicity of chitosan, research on antioxidant activity of chitosan and its derivatives will be helpful to expand their application in foods and medicinal materials. In this study, we attempt to degrade chitosan by cellulase to enhance its free radical scavenging activity. The products, with different molecular weight, were comparatively investigated by GPC, FT-IR, XRD, and NMR. The relation between the free radical scavenging activity and molecular weight of chitosan was discussed.

EXPERIMENTAL

Materials

Chitosan with a weight-average molecular weight (Mw) of 390 kDa and the degree of DD of 82.8% was obtained from Yuhuan Biochemical Co. (Zhejiang, China). Nitroblue tetrazolium (NBT) was purchased from Sigma Chemical Co. All other chemicals and reagents used were of analytical grade.

The cellulase, a commercial enzyme, was a product of Ningxia XiaSheng Industry Co., Ltd. (China).

Preparation of samples by enzymatic hydrolysis

Chitosan (20 g) was completely dissolved in 500 mL 1% acetic acid. Then the solution was adjusted to pH 5.6. The solution in the reaction vessel was placed in a thermostatic water bath at 55°C and a solution in which the cellulase (0.8 g) was dissolved in 0.2*M* acetate buffer (pH 5.6) was added to initiate reaction. After 1, 2, and 4 h, the mixture was taken out respectively, and boiled for 10 min to remove the enzyme. After filtering, the filtrated was concentrated with a rotary evaporator under reduced pressure followed by neutralization with 10% KOH to pH 9, and then precipitated by adding anhydrous ethanol. The precipitated was collected by filtration, and washed thoroughly with anhydrous ethanol. The LMWCs were collected after drying over phosphorus pentoxide in vacuum.

Characterization

Weight-average molecular weight (M_w), number-average molecular weight (M_n) and molecular weight dispersion (M_w/M_n) of sample were measured by GPC. The GPC equipment consisted of connected columns (TSK G3000-PW), TSP P100 pump and RI 150 refractive index detector. The eluent was 0.2*M* CH_3COOH/0.1*M* CH_3COONa. The flow rate was maintained at 1.0 mL min^{-1}. The standards used to calibrate the column were pullulan purchased from Showa Denko, Tokya, Japan.

FT-IR spectra were recorded with KBr pellets on a Nicolet FT-IR 5700 spectrophotometer. Sixteen scans at a resolution of 4 cm^{-1} were averaged and referenced against air.

X-ray diffraction patterns of the degraded chitosan fractions were measured by a diffractometer type D/max-rA (Tokyo, Japan) with Cu target and Kα radiation at 40 kV and 50 mA at 20°C. The relative intensity was recorded in the scattering range (2θ) of 5–40°.

NMR spectra were recorded on a Varian mercury 300 spectrometer. LMWC3 was dissolved in D_2O.

Potentiometric determination of the degree of DD

The chitosan (0.1 g) was dissolved in a known excess of 0.1*M* HCl acid (10 mL). By the titration of this solution with a 0.1*M* NaOH solution, a curve with two inflection points was obtained. The amount of the acid consumed between these two points was considered to correspond to the amount of the free amino groups in the solution.[9] The titration was performed with a DELTA-320-S pH meter.

Estimation of water-solubility

The pH dependence of water solubility of chitosan was evaluated from turbidity. Chitosan (0.1 g) was dissolved 1% HAc (100 mL). The transmittance of the solution was recorded with the stepwise addition of concentrated NaOH, on a Shimadzu UV-9100 spectrophotometer using a quartz cell with an optical path length of 1 cm at 600 nm.

Assay of superoxide radical scavenging activity

The assay was based on the capacity of chitosan to inhibit the photochemical reduction of NBT in the riboflavin-light-NBT system.[10] The method was used by Dasgupta[11] for determination of antioxidant activity of *Piper betle L.* leaf extract. Reagent mixture (3 mL), containing 50 m*M* sodium phosphate buffer pH 7.8, 13 m*M* methionine, 2 μ*M* riboflavin. EDTA (100 μ*M*) and 75 μ*M* NBT, was mixed with chitosan sample solution (1 mL). The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after a 10-min illumination from a fluorescent lamp. The entire reaction assembly was enclosed in a box lined with aluminum foil. Identical tubes with reaction mixture were kept in the dark and served as blanks.

$$\%\text{Inhibition} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance of samples.

TABLE I
The Molecular Parameters of Cellulase-Treated Chitosan

Sample	Reaction time (h)	M_w ($\times 10^{-3}$)	DD (%)	M_w/M_n	Yield (%)
chitosan	0	390	82.8	6.73	–
LMWC1	1	27.3	81.3.	3.02	91.7
LMWC2	2	5.9	80.5	1.87	84.3
LMWC3	4	1.7	80.8	1.31	74.6

Assay of hydroxyl radical scavenging activity

The assay was based on benzoic acid hydroxylation method, as described by Chung et al.[12] In a screw-capped tube, 10 m*M* sodium benzoate (0.2 mL), 10 m*M* $FeSO_4$ (0.2 mL), and 10 m*M* EDTA (0.2 mL) were added into each tube. Then the sample solution (0.2 mL) and 0.1*M* sodium phosphate buffer pH 7.4 (1 mL) were added into the tube to give a total volume of 1.8 mL. Finally, 10 m*M* hydrogen peroxide solution (0.2 mL) was added to the reaction mixture which was then incubated at 37°C for 2 h. After this, the fluorescence was measured at 407 nm emission with excitation at 305 nm.

$$\%\text{Inhibition} = [1 - (F_1 - F_0)/(F_2 - F_0)] \times 100$$

where F_0 is fluorescence intensity with no treatment, F_2 is fluorescence intensity of treated control, F_1 is fluorescence intensity of treated sample.

RESULTS AND DISCUSSION

The molecular parameters of cellulose-treated chitosan

The optimum pH and temperature for depolymerizing chitosan by cellulase were determined at pH 5.6 and 55°C, respectively. The molecular parameters of cellulose-treated chitosan were listed in Table I. Obviously, prolonging the duration increased the extent of degradation, and the degradation of the backbone mainly occurred in a random fashion because of a rapid decrease of molecular weight in the beginning. With the decreasing of molecular weight, the molecular weight distribution (M_w/M_n) decreased. The degree of DD of the low molecular weight chitosan did not change with the decrease of molecular weight.

FT-IR spectra

FT-IR spectroscopy has been shown to be a powerful tool for the study of the physicochemical properties of polysaccharides. Curves a, b, and c in Figure 1 show the IR spectra of initial chitosan and LMWC1, LMWC3. The absorption bands at 1653, 1598, 1322 cm^{-1} in initial chitosan are attributed to the amide I band, *N*—H bending mode of $-NH_2$ and amide III band, respectively. The spectrum of LMWC1 and LMWC3 are similar to that of initial chitosan, but the amide I band shifts to low wave number, it suggested that carbonyl groups had more opportunity to form stronger hydrogen bonds in that the scission of polymer chains led to the increasing mobility of molecules and with the decrease of the molecular weight of chitosan. The IR spectra suggested that there was no significant difference between the residues of chitosan before and after the enzymatic hydrolysis.

X-ray analysis

Six polymorphs have been proposed for chitosan: "tendon," "annealed," "*l*-2," "L-2," "form-I," and "form-II."[13] Figure 2 shows the X-ray diffraction patterns of the main fraction of resulting chitosan by the progress of degradation. The wide-angle X-ray diffraction (WAXD) pattern of initial chitosan shows its characteristic peaks at $2\theta = 10.4°$ and $20.2°$, which coincided with the pattern of the "L-2 polymorph" of chitosan previously reported.[14] LMWC1 displayed a new characteristic peak at $2\theta = 21.8°$, and for the LMWC1 the intensity of the characteristic peak at $2\theta = 20.2°$ increased. Both of the two patterns characterized a chitosan polymorph, which is referred to as the "tendon hydrate polymorph."[15] LMWC2

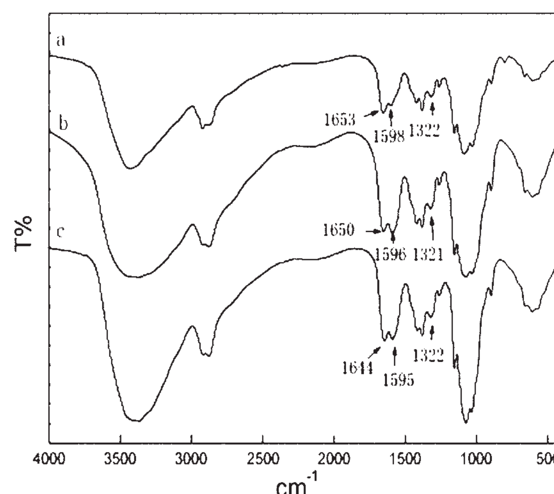

Figure 1 FT-IR spectra of chitosan (a) and degraded chitosan LMWC1 (b), LMWC3 (c).

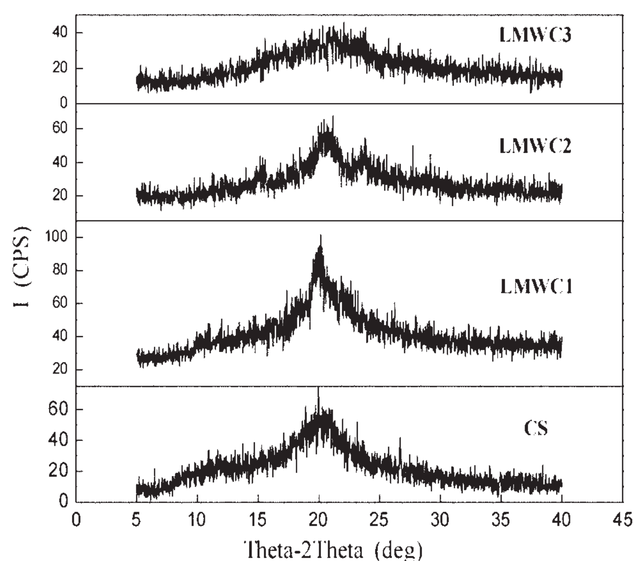

Figure 2 X-ray diffraction patterns of initial chitosan and degraded chitosans.

displayed the other two new characteristic peaks at $2\theta = 15.1°$ and $23.8°$, which is referred to as the "annealed polymorph," just as previously described by Ogawa,[16] and then was found to be a mixture of the "tendon hydrate polymorph" and "annealed polymorph." As the extent of conversion from the "tendon chitosan" to annealed form depends upon the molecular weight of chitosan, LMWC2 did not show complete conversion to the annealed polymorph because of the lower mobility of its polymer chains. As a consequence, there seems to be an obvious correlation between the depolymerization and the rise in crystallinity due to recrystallization of short chain chitosan. LMWC3 had only one major peak and became amorphous.[17] Therefore, the chitosan in amorphous region was first degraded to water-soluble molecules, and dissolved in water. With deeper degradation, the crystalline structure was destroyed.

NMR

To further confirm the structure of the degraded chitosan, the water-soluble product LMWC3 was analyzed by NMR spectroscopy in D_2O.

Figure 3 shows the ^1H NMR spectrum of LMWC3 measured in D_2O solution at ambient temperature. The resonance at 1.95 ppm is easily assigned to the three *N*-acetyl protons.[18] The peak at 4.5–4.7 ppm and the peak at 2.6–2.9 ppm correspond to H-1 and H-2 of the units, respectively.[19] The resonance of H-3 of GlcN residue was at 3.55 ppm while the resonance of H-3 of ClcNAc was at 3.46 ppm. No attempts were made to identify other peaks, because the spectra of partially unfractionated *N*-deacetylated chitooligomers are very difficult to be interpreted. Nevertheless, all signals were the resonances of oligomers of chitin and chitosan, which coincided with the reported data.[20]

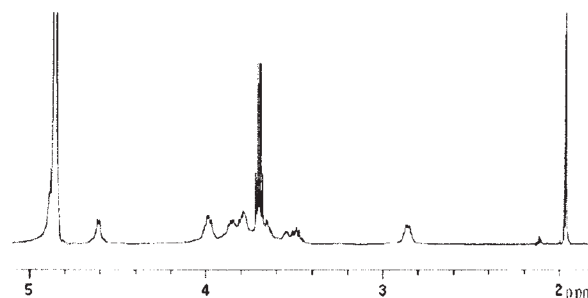

Figure 3 The ^1H NMR spectrum of LMWC3.

The ^{13}C NMR spectrum of LMWC3 measured in D_2O solution at ambient temperature was shown in Figure 4. The strong signals at 56.0, 59.8, 73.0, 74.5, 77.5, and 101.3 ppm have been attributed to C-2, C-6, C-3, C-5, C-4, and C-1, respectively. The assignments of signals are based on data found in the literature.[20] The complexity of the spectrum arises due to sequences-dependent shifts of carbons, and the formation of the new reducing and nonreducing ends. The ^{13}C peak of C=O at 181 ppm in LMWC3 indicates the partially acetylation of LMWC3. Although we have not attempted to assign all the carbon signals of unfractionated partially *N*-deacetylated chitooligomers, all signals belong to chitooligomers according to previous literature.

Solubility of degraded chitosan

Figure 5 shows the pH dependence of the transmittance of the chitosan solution. In the cases of the lower-molecular-weight chitosan, the water solubility was high and retained over a wide pH range, whereas in the cases of the higher-molecular-weight chitosan, it was high at acidic pH but abruptly decreased at a pH a little over neutrality. Especially, LMWC3 gave very high solubility but the solubility of rest decreased with increasing molecular weight in the alkaline region. It seems that the high water solubility of low-molecular-weight chitosan is attributed to the decrease of intermolecular interactions,

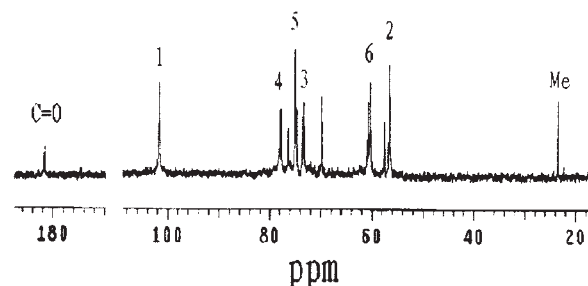

Figure 4 The ^{13}C NMR spectrum of LMWC3.

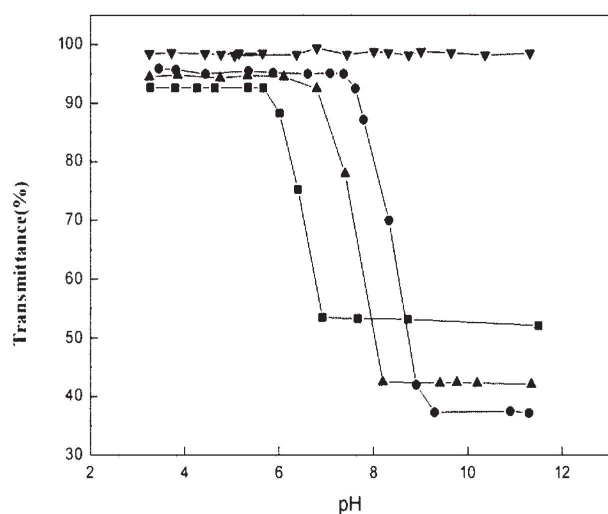

Figure 5 pH dependence of water solubility of degraded chitosan. (■) chitosan, (▲) LMWC1, (●) LMWC 2, (▼) LMWC 3.

such as van der Waals forces; the lower the molecular weight, the lower the intermolecular attraction forces.[19] And when the chitosans were dissolved in aqueous acetic acid, their solubility at neutral pH appears to be higher than that in pure water. The ionic strength might be a cause for this phenomenon.

Free radical scavenging activity

Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. It is an initial free radical formed from mitochondrial electron transport systems. Superoxide anion radical is produced by a number of cellular reactions, including various enzyme systems, such as lipoxygenases, peroxidase, NADPH oxidase, and xanthine oxidase. They play an important role in the formation of other cell-damaging free radicals and molecules, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems.[21] Hydroxyl radical is the most reactive-free radical, and can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions, such as copper or iron. They can react with nonselective compounds such as proteins, DNA, unsaturated fatty acids, and almost every biological membrane. Thus, superoxide anion radical and hydroxyl radical quenching assay were used for the evaluation of radical scavenging activity of cellulase treated chitosan *in vitro*.

Figure 6 shows the superoxide radical scavenging activity of different molecular weight of cellulase-treated chitosan at various concentrations. Significant scavenging of superoxide radical was evident at all the tested concentration of LMWC3. As shown in Figure 6, at 0.1 mg mL^{-1}, scavenging percentage of LMWC3 against superoxide radical was 79.3%. Scavenging activity of low molecular weight chitosan against superoxide radical was more pronounced than that of high molecular weight chitosan. Figure 7 shows the hydroxyl radical scavenging activity of cellulase treated chitosan. At 2.0 mg mL^{-1}, scavenging percentage of initial chitiosan, LMWC1, LMWC2, and LMWC3 against hydroxyl radical was 14.3%, 33.1%, 47.4%, and 65.9%, respectively. Obviously, low molecular weight chitosan exhibits higher hydroxyl radical scavenging activity.

Chtiosan has two hydroxyl groups and one amino group in its monosaccharide construction unit. The hydroxyl groups in the polysaccharides unit can react with free radical by the typical H-abstraction

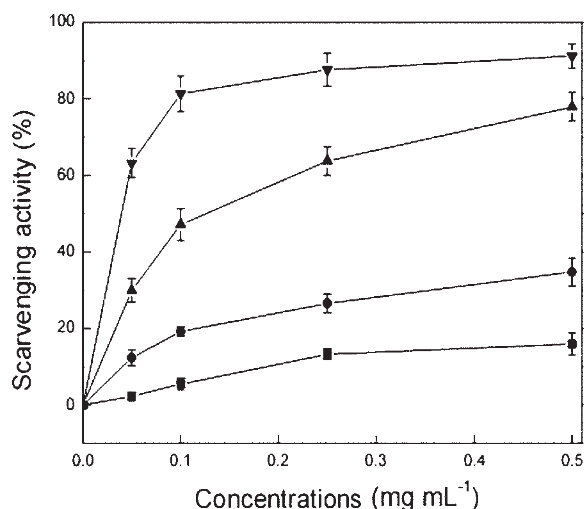

Figure 6 Superoxide radical scavenging activity of degraded chitosan. (■) chitosan, (●) LMWC1, (▲) LMWC 2, (▼) LMWC 3.

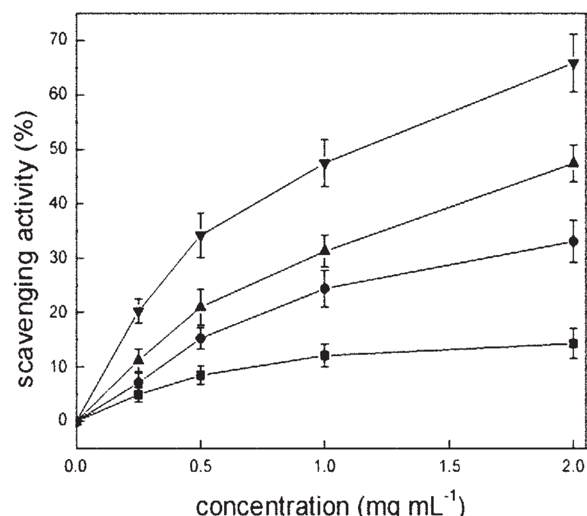

Figure 7 Hydroxyl radical scavenging activity of degraded chitosan. (■) chitosan, (●) LMWC1, (▲) LMWC 2, (▼) LMWC 3.

reaction.[22] On the other hand, the scavenging mechanism of chitosan is related to the fact that free radical can react with the residual-free amino groups NH_2 to form stable macromolecule radicals, and the NH_2 groups can form NH_3^+ groups by absorbing a hydrogen ion from the solution.[23] Therefore, the active hydroxyl and amino groups in the polymer chains are the origin of the scavenging ability of chitosan. As shown in Figures 6 and 7, scavenging activity of low molecular weight chitosan against superoxide radical and hydroxyl radical were more pronounced than that of high molecular weight chitosan. This may be the effect of intramolecular hydrogen bond.[24] High molecular weight chitosan has a compact structure and the effect of intramolecular hydrogen bond is stronger. The strong effect of the intramolecular hydrogen bond weakens the activities of the hydroxyl and amino groups, and the chance of exposure of their hydroxyl and amine groups might be restricted which would account for less radical scavenging activity. And hydroxyl radical scavenging activity of chitosan can be partially attributed to its metal chelating ability. The Fe^{2+} chelating ability of chitosan mainly comes from the presence of amino groups, which contain lone electron pairs that help to form chitosan- Fe^{2+} complexes.[25]

CONCLUSIONS

A cheap, commercially available, and efficient cellulase was used to degrade partially *N*-acetylated chitosan. The reduction in molecular weight of the resulting chitosan led to transformation of crystal structure and increase of water-solubility. FT-IR and NMR spectra confirmed that the chemical structures of residues were not modified. Low molecular weight chitosan samples exhibited high scavenging activity against superoxide anion radical and hydroxyl radical, and they have promising application in food for human health.

References

1. Kumar, M. N. V. R. React Funct Polym 2000, 46, 1.
2. Shahidi, F.; Arachchi, J. K. V.; Jeon, Y. J Trends Food Sci Tech 1999, 10, 37.
3. Kim, S. K.; Rajapakse, N. Carbohydr Polym 2004, 62, 357.
4. Liu, J; Xia, W. S. Biochem Eng J 2006, 30, 82.
5. Li, J.; Du, Y.; Liang, H. J Appl Polym Sci 2006, 102, 1098.
6. Wanita, A.; Lorenz, K. J Food Process Pres 1996, 20, 917.
7. Je, J. Y.; Park, P. J.; Kim, S. K. Food Chem Toxicol 2004, 42, 381.
8. Park, P. J.; Je, J. Y.; Kim, S. K. Carbohydr Polym 2004, 55, 17.
9. Tolaimate, A.; Desbrieres, J.; Rhazi, M.; Alagui, A.; Vincendon, M.; Vottero, P. Polymer 2000, 41, 2463.
10. Beauchamp, C.; Fridovich, I. Anal Biochem 1971, 44, 276.
11. Dasgupta, N.; De, B. Food Chem 2004, 88, 219.
12. Chung, S. K.; Osawa, T.; Kawakishi, S. Biosci Biotech Bioch 1997, 61, 118.
13. Samuels, R. J. J Polym Sci Polym Chem 1981, 19, 1081.
14. Saito, H.; Tabeta, R.; Ogawa, K. Macromolecules 1987, 20, 2424.
15. Belamie, E.; Domard, A.; Giraud-Guille, M. M. J Polym Sci Pol Chem 1997, 35, 3181.
16. Ogawa, K. Agric Biol Chem 1991, 55, 2375.
17. Kurita, K.; Sannan, T.; Iwakura, Y. Makromol Chem 1977, 178, 3197.
18. Akiyama, K.; Kawazu, K.; Kobayashi, A. Carbohydr Res 1995, 279, 151.
19. Kubota, N.; Tatsumoto, N.; Sano, T.; Toya, K. Carbohydr Res 2000, 324, 268.
20. Qin, C.; Du, Y.; Zong, L.; Zong, F.; Liu, Y., Zhou, B. Polym Degrad Stabil 2003, 80, 435.
21. Bloknina, O.; Virolainen, E.; Fagerstedt, K. V. Ann Bot-London 2003, 91, 179.
22. Xue, C.; Yu, G.; Hirata, T.; Terao, J.; Lin, H. Biosci Biotech Bioch 1998, 62, 206.
23. Xie, W.; Xu, P.; Liu, Q. Bioorg Med Chem Lett 2001, 11, 1699.
24. Xing, R.; Liu, S.; Guo, Z.; Yu, H.; Wang, P.; Li, C.; Li, Z.; Li, P. Bioorgan Med Chem 2005, 13, 1573.
25. Guzman, J.; Saucedo, I.; Revilla, J.; Navarro, R.; Guibal, E. Int J Biol Macromol 2003, 33, 57.