Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Polymers 64 (2006) 553–559
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Effect of ultrasonic treatment on the biochemphysical properties of chitosan

Hui Liu [a,b,*], Jianguo Bao [a,b], Yumin Du [c], Xuan Zhou [a], John F. Kennedy [d,e]

[a] School of Environmental Studies, China University of Geosciences, Wuhan 430074, China
[b] Key Laboratory of Biological and Environmental Geology, China University of Geosciences, Wuhan 430074, China
[c] Department of Environmental Science, Wuhan University, Wuhan 430072, China
[d] Birmingham Carbohydrate and Protein Technology Group, School of Chemical Sciences, University of Birmingham, Birmingham B15 2TT, UK
[e] Chembiotech Laboratories, University of Birmingham Research Park, Vincent Drive, Birmingham B15 2SQ, UK

Received 30 July 2005
Available online 13 December 2005

Abstract

Four chitosans with different molecular weights and degrees of deacetylation degree and 28 chitosans derived from these initial chitosans by ultrasonic degradation have been characterized by gel permeation chromatography (GPC), FT-IR spectroscopy, X-ray diffraction and titrimetric analyses. Antimicrobial activities were investigated against *E. coli* and *S. aureus* using an inhibitory rate technique. The results showed that ultrasonic treatment decreased the molecular weight of chitosan, and that chitosan with higher molecular weight and higher DD was more easily degraded. The polydispersity decreased with ultrasonic treatment time, which was in linear relationship with the decrease of molecular weight. Ultrasonic degradation changed the DD of initial chitosan with a lower DD (<90%), but not the DD of the initials chitosan with a higher DD (>90%). The increased crystallinity of ultrasonically treated chitosan indicated that ultrasonic treatment changed the physical structure of chitosan, mainly due to the decrease of molecular weight. Ultrasonic treatment enhanced the antimicrobial activity of chitosan, mainly due to the decrease of molecular weight.

Keywords: Chitosan; Ultrasonic; Molecular weight; Deacetylation degree; Antimicrobial activity

1. Introduction

Chitosan is a linear copolymer of 1,4-linked 2-amino-2-deoxy-β-D-glucopyranose (GlcN) and 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) units. It has received much attention as a functional biopolymer for many diverse applications in food (Shahidi, Arachchi, & Jeon, 1999), pharmaceutics (Dodane & Vilivalam, 1998), agriculture, cosmetics (Kumar, 2000), due to its biological activities such as antimicrobial (Choi et al., 2001; Jeon, Park, & Kim, 2001; No, Park, Lee, & Meyers, 2002; Roller & Covill, 1999), antitumour (Suzuki et al., 1986) and immune enhancing effects (Sugano, Yoshida, Ilashimoto, Enomoto, & Ilirano, 1992). These functions have been revealed to be dependent not only upon the chemical structure but also the molecular size of the chitosans.

Recent research showed that low molecular weight chitosans with weight average molecular weights in the range of 5–10 KDa exhibited strong bactericidal and superior biological activities compared to chitosan with high molecular weight (Kittur, Vishu Kumar, & Tharanathan, 2003). Low molecular weight chitosansof 20 kDa were shown to prevent progression of diabetes mellitus and exhibit higher affinity for lip polysaccharide than 140 kDa chitosan (Kondo, Nakatani, Hayashi, & Ito, 2000). Low molecular weight chitosans of 5–10 KDa were shown to have potential as a DNA delivery system (Richardson, Kolbe, & Duncan, 1999). Both low molecular weight chitosans and chito-oligomers exhibited special antimicrobial activity in other tests (Vishu Kumar, Varadaraj, Lalithab, & Tharanathan, 2004; Zheng & Zhu, 2003; No, Park, Lee, & Meyers, 2002; Begona & Ruth, 1997) and antitumour activity (Qin, Du, Xiao, Li, & Gao, 2002b; Seo, Pae, & Chung, 2000). So it is of increasing interest to degrade chitosan to low molecular weight under appropriate conditions and then compare the relationship between biological activity and molecular weight.

The methods for preparing low molecular weight chitosans and chito-oligomers can be chemical, enzymatic or physical.

* Corresponding author. Tel.: +86 27 67883152; fax: +86 27 87436235.
E-mail address: jhzhou168@163.com (H. Liu).

0144-8617/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2005.11.007

The chemical treatment such as hydrogen peroxide degradation (Qin, Du, and Xiao, 2002a) is a very common and fast method for producing a series of the chitosan oligomers, but this procedure has some faults such as high cost, low yield and environmental pollution. The enzymatic method, such as with chitosanase (Jeon, Park, & Kim, 2001), proteases (Li et al., 2005), cellulose (Qin et al., 2004) and hemicellulose (Qin et al., 2003) seems to be generally preferable to chemical reactions because the reaction course is under gentle conditions and product distribution can be controlled more readily. However, the expensive cost of the specific enzymes such as chitosans and chitinase inhibits their use on an industrial scale. Physical methods, such as sonication (Czechowaska-Biskup, Rokita, Lotfy, Ulanski, & Janusz, 2005; Cravotto, Tagliapietra, Robaldo, & Trotta, 2005; Chen, Chang, & Shyur, 1997; Wang and Qin, 1989; Muzzarelli & Rocchetti, 1985) and irradiation (Choi, Ahn, Lee, Byun, & Park, 2002; Lim, Khor, & Koo, 1998) are energy saving, environmentally friendly and effective. The principle behind the physical means is to provide the added energy needed to break the chemical bonds.

The effects of ultrasonic conditions on the degradation of chitosan have been reported by several workers. Chen, Chang, and Shyur (1997) studied the effect of ultrasonic conditions, including parameters of chitosan concentration, reaction temperature, type of solvent and ultrasonic time and storage in acidic solution, on changes in the molecular weight and polydispersity (molecular weight distribution) of treated chitosan. Results showed that chitosan was degraded faster in dilute solutions than in concentrated solutions and faster in lower temperature solutions than in higher temperature solutions. Degradation increased with prolonged ultrasonication time. The polydispersity decreased with ultrasonic treatment for all ultrasonic conditions. Ultrasonic degradation rate was also affected by DD. Tsaih & Chen (2003) revealed that the degradation rate and rate constant increased with an increasing DD of the chitosan used. However, Trzciński & Staszewska (2004) showed that the general rate parameter (k) increased with the degree of N-acetylation. The effects of ultrasonic conditions on changes of deacetylation degree (DD) of treated chitosan have been studied, but the results are sometimes controversial. Some researchers considered that ultrasonic treatment does not change the DD of the treated chitosan (Chen & Tsaih, 1998; Tsaih & Chen, 1997; Chen & Hwa, 1996; Wang, Bo, Li, & Qin, 1991; Wang & Lin, 1989). However, Muzzarelli & Rocchetti (1985) reported the sonification leads to immediate chain degradation and to detectable deacetylation after more prolonged periods of treatment, especially at a pH of 1.0.

The effects of the molecular weight and DD on the molecular parameters, such as molecular weight, polydispersity, DD, physical characteristics of ultrasonic degraded chitosan resultants have rarely been studied. So in this paper, four chitosans with different molecular weight and DD have been degraded by ultrasonication, and 28 degraded chitosan resultants were gained. All 32 chitosan samples were characterized by gel permeation chromatography (GPC), FT-IR, X-ray diffraction, titration, to study the above effects. Due to the difference among the degradation mechanisms, low molecular weight chitosans prepared by different method may show different biological activity. Until now, there is no report about the biological properties of ultrasonic degraded chitosan, so the antimicrobial activity of 14 chitosan samples was also investigated.

2. Experimental

2.1. Materials

Chitosan, as initial materials from shrimp shells, was purchased from Yuhuan Ocean Biochemical Co. (Zhejiang, China). The molecular parameters of the initial chitosan are listed in Table 1. All other chemicals are of reagent grade.

2.2. Ultrasonic degradation of chitosans with different deacetylation degrees and molecular weights

The initial chitosan samples with different deacetylation degree and molecular weight listed in Table 1 were ultrasonically degraded according to the reference (Chen & Hwa, 1996). The chitosan samples were dissolved in 2% acetic acid and then ultrasonically degraded for 0, 6.0, 16.5, 32.5, 50.5, 68.5, 86.0, 99.0 h using a CGT-600 sonicator (Zhangjiagang Gangwei, Jiangsu, China) at an energy level of 250 W at 80 °C. The degraded solutions were neutralized with 0.1 M NaOH to precipitate the degraded chitosans. Some chitosans with low molecular weight may not be precipitated in this process because they are soluble in alkaline solution. They were collected and washed with distilled water until neutral then dried at 50 °C thereby yielding chitosans with different molecular weights.

2.3. Characterizations

Weight average molecular weight (M_w), number average molecular weight (M_n) and molecular weight dispersion (M_w/M_n) of samples were measured by GPC. The GPC equipment consisted of connected columns (TSK G5000-PW and TSK G3000-PW), TSP P100 pump and RI 150 refractive index detector. The eluent was 0.2 M acetic acid/0.1 M sodium acetate buffer pH 4.8. Eluent and chitosan sample (0.4 mg/ml) solutions were filtered through 0.45 μm Millipore filters. The flow rate and temperature were maintained at 1.0 ml/min and 30 °C, respectively. The standards used to calibrate the column were TOSOH pullulan. All data provided by the GPC system were collected and analyzed using the Jiangshen Workstation software package (DaLian Jiangshen, DaLian, China).

The deacetylation degrees of chitosans were determined according to previous works (Li et al., 2005). The chitosan (0.1 g) was dissolved 0.1 M hydrochloric acid (10 ml). From

Table 1
The molecular parameters of the initial chitosans

Initial chitosan	CS 1-0	CS 2-0	CS 3-0	CS 4-0
M_w ($\times 10^4$ kDa)	65.5	154.0	77.5	44.7
DD (%)	61.9	72.1	87.1	91.6

the titration of this solution with a DELTA-320-S pH meter with a 0.1 M NaOH solution, a curve with two inflexion 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. The titration was performed.

FT-IR spectra were recorded in KBr pellets on a Nicolet FT-IR 360 spectrophotometer. Sixteen scans at a resolution 4 cm^{-1} were averaged and referenced against air.

X-ray diffraction patterns were measured by a Shimadzu Lab XRD-6000 diffractrometer using a Cu Kα target at 40 KV and 50 mA at 20 °C. The relative intensity was recorded in the scattering range (2θ) of 5–50°.

2.4. Assays for antimicrobial activity

E. coli ATCC 25922, Staphylococcus aureus ATCC 25923 for antimicrobial assay were provided by the Typical Cultural Collection Center in Wuhan University, China. Bacteria were incubated on nutrient agar (peptone 1%, beef extract 0.5%, NaCl 0.5%, agar 2%, pH 6) at 37 °C for 1 day. To prepare bacterial suspensions, colonies of bacteria on agar plates were transferred into sterile saline (0.9% w/v) solution, then diluted to obtain a bacterial suspension containing 10^5–10^6 cells/ml.

1% w/v chitosan solution in acetate buffer (20 μl) or acetate buffer (20 μl) itself was added to the mixture of the prepared bacterial suspension (20 μl) and the meat juice medium (peptone 1%, beef extract 0.5%, NaCl 0.5%, pH 6), and was incubated with shaking at 37 °C for 20 h. The turbidity of the resultant medium was measured at 640 nm using a 1601 UV–vis spectrophotometer (Shimadzu, Tokyo, Japan).

The inhibitory effect of chitosan was indicated by the inhibitory rate that was calculated by the following equation:

$$\text{Inhibitory rate} = 1 - \frac{A_{cs} - A_{cs0}}{A_{buffer} - A_{buffer0}} 100\%$$

Where

A_{cs} the absorbance of the bacteria medium with chitosan after incubated,

A_{cs0} the absorbance of the bacteria medium with chitosan before incubated,

A_{buffer} the absorbance of the bacteria medium with acetate buffer after incubated, and

$A_{buffer0}$ the absorbance of the bacteria medium with acetate buffer before incubated.

3. Results and discussion

3.1. Effect of ultrasonic treatment on the molecular weight of chitosans

In the GPC profiles of chitosans degraded ultrasonically (Fig. 1), the shift toward higher elution times as a consequence of the degradation could be observed for the four samples. The molecular weights of 32 chitosan samples derived from ultrasonic degradation of 4 chitosans for different times are shown in Fig. 2. Molecular weights decreased rapidly with increasing ultrasonic depolymerization time during the first period, and then the increase gradually slowed down during the remaining treatment time for all four chitosans studied. Furthermore, over the same time span of 99 h, the degradation degree of CS 2-0, which has a higher molecular weight, was higher than that for other chitosans. These results indicate that chitosan with higher molecular weight is easier to be degraded.

It has been reported that the curves of molecular weight of degraded chitosan versus the ultrasonic (300 W) time showed an inflexion at 1h treatment (Chen et al., 1997; Tsaih & Chen, 2003). This may be because the chances of being attacked by the capitation energy increased with increasing molecular weight species and may be because smaller molecular weight species have shorter relaxation times and, thus, can resist the sonication stress more easily.

The degree of ultrasonic degradation of chitosan was also affected by the DD. The curve of CS 4-0 with high DD showed an inflexion at the first 6-h, however, this inflexion gradually disappeared with decrease of DD (Fig. 2). This result indicates that chitosan with higher DD was easier to be degraded in short

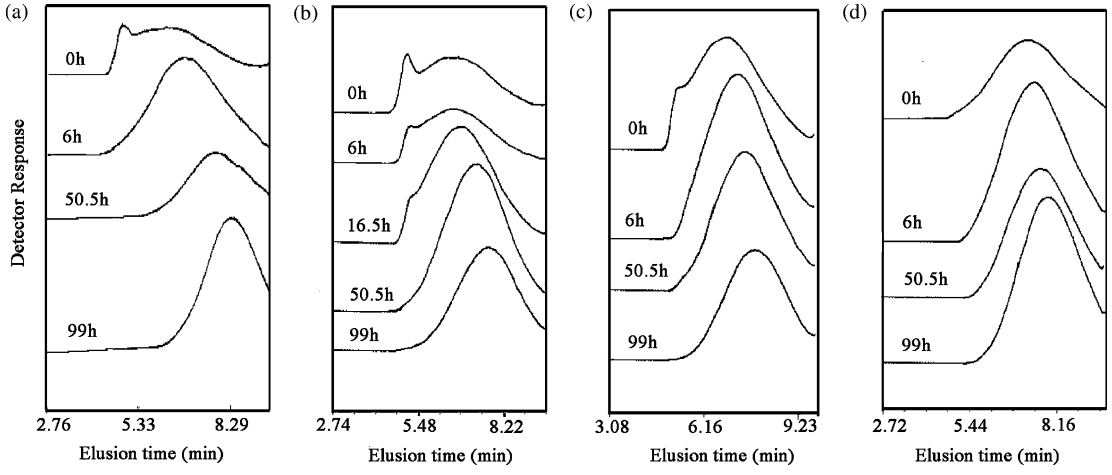

Fig. 1. GPC profiles of (a) CS1-0, (b) CS2-0, (c) CS3-0, (d) CS4-0 and their ultrasonic degraded products at different depolymerization time.

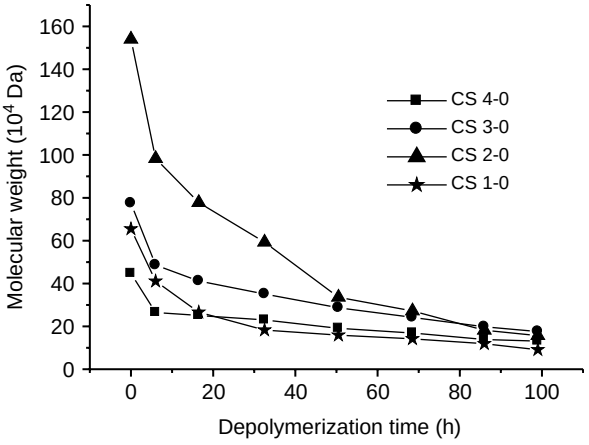

Fig. 2. Molecular weight of ultrasonic degraded chitosan versus depolymerization time.

time, which may be because the more flexible molecules of higher DD chitosans are more susceptible to the shear force of elongation flow generated by the capitation field or due to the different bond energies of acetamido, 1,4-β-D-glucosidic linkages, and hydrogen bonds (Tsaih & Chen, 2003).

3.2. Effect of ultrasonic treatment on the polydispersity of chitosans

The increasingly narrower peaks of chitosans with prolonged ultrasonic treatment time (Fig. 1) indicated that the molecular weight tends towards being homogeneous. In the GPC profiles of CS 1-0, CS 2-0 and CS 3-0, there is a smaller peak next to the left of the main peak, which is probably due to the aggregation of the chitosan molecules; this gradually disappeared with degradation time. Besides, the size of small peak is related to the molecular weight; the higher the molecular weight is, the larger the small peak.

To study the polydispersity more carefully, the M_w/M_n values of chitosans were calculated; The M_w/M_n values of chitosans derived from ultrasonic degradation of CS 2-0, CS 3-0 and CS 4-0 for different times obviously decreased with depolymerization times (Fig. 3). This result is agreement with Chen et al. (1997). Besides, the M_w/M_n of CS 2-0 with higher molecular weight decreased with degradation time more rapidly, which indicates that the polydispersity is related to the molecular weight. Further study revealed that polydispersity and molecular weight of chitosan exhibited a liner relationship (Fig. 4(a)). However, there was no good linear relationship between polydispersity and DD (Fig. 4(b)). So the decrease of chitosan polydispersity is mainly due to the decrease of molecular weight, not the degradation methods, because this also can be caused by other methods such as enzymatic hydrolysis (Li et al., 2005).

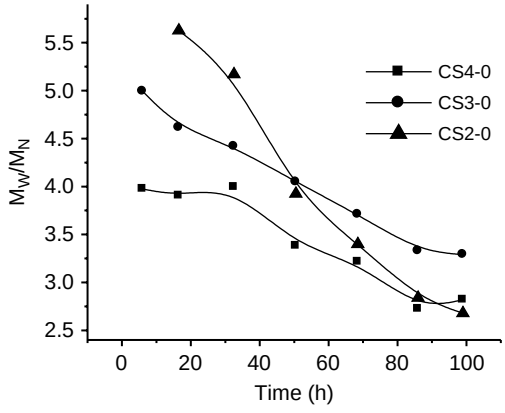

Fig. 3. Polydispersity (M_w/M_n) of chitosan products at different depolymerization times.

3.3. Effect of ultrasonic treatment on the deacetylation degree of chitosans

Wang & Qin (1989) reported that the DD of chitosan did not change with ultrasonic treatment. Then some researchers used this method to prepare chitosan samples with same DD (Chen & Tsaih, 1998; Tsaih & Chen, 1997; Chen & Hwa, 1996). However, Muzzarelli & Rocchetti (1985) reported that sonification leads to immediate chain degradation and to detectable deacetylation after more prolonged periods of treatment, especially at a pH of 1.0. So in this work the DD of every chitosan sample was determined. Surprisingly, Fig. 5 shows that ultrasonic treatment almost did not change the DD of chitosan with initial high DD (>90%). This result is in

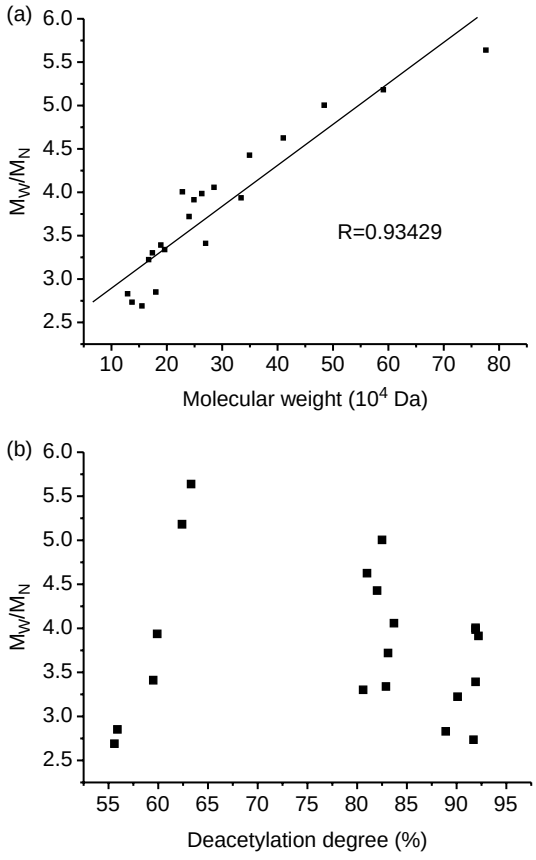

Fig. 4. The relationship between polydispersity (M_w/M_n) and molecular weight (a), deacetylation degree (b) of ultrasonic degraded chitosans.

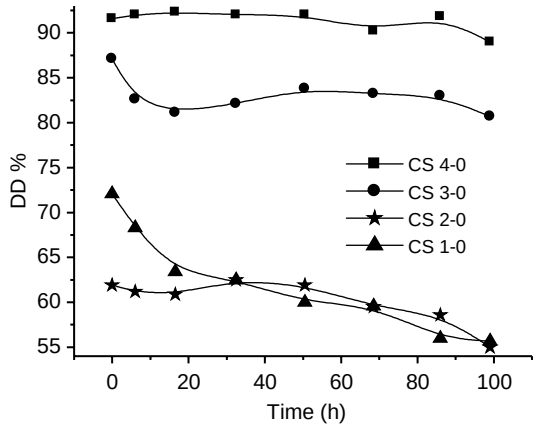

Fig. 5. Dacetylation degree of chitosan products at different depolymerization times.

accordance with Wang & Qin (1989). But for the initial chitosan with lower DD (<90%), the DD of ultrasonic treated chitosan decreased obviously, especially with initial chitosans with DD lower than 80%. In 99 h, the DD of CS2-0 and CS1-0 decreased 16.4 and 6.9%, respectively. So the difference between the DD's of initial chitosans can explain the diversity of the reports of Wang & Qin (1989) and Muzzarelli & Rocchetti (1985).

The DD decrease of chitosan with ultrasonic treatment time can also be seen in the IR spectra (Fig. 6). The absorption bands at 1663, 1554 and 1321 cm^{-1} are, respectively, referenced as amide I, II and III bands, and the absorption band at 1601 cm^{-1} is ascribed to N–H bending mode in the primary amine (Qin et al., 2002a,b). The relative absorption intensity of $–NH_2$ in the derived chitosans is CS 2-0 > CS2-5 > CS2-7 and CS3-0 > CS3-7, suggesting that the DD decreased with advance of ultrasonic degradation time. This result coincided well with the DD of ultrasonically treated chitosan with an initial DD lower than 80% decreasing with advance of ultrasonication time (Fig. 5).

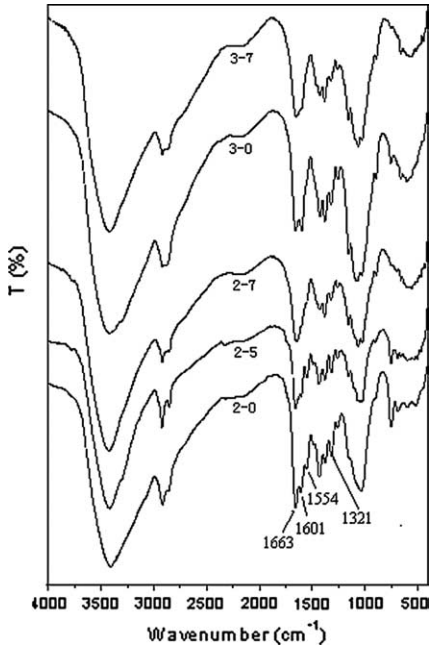

Fig. 6. IR spectra of initial chitosans CS2-0, CS3-0 and their ultrasonic treated products CS2-5, CS2-7 and CS3-7.

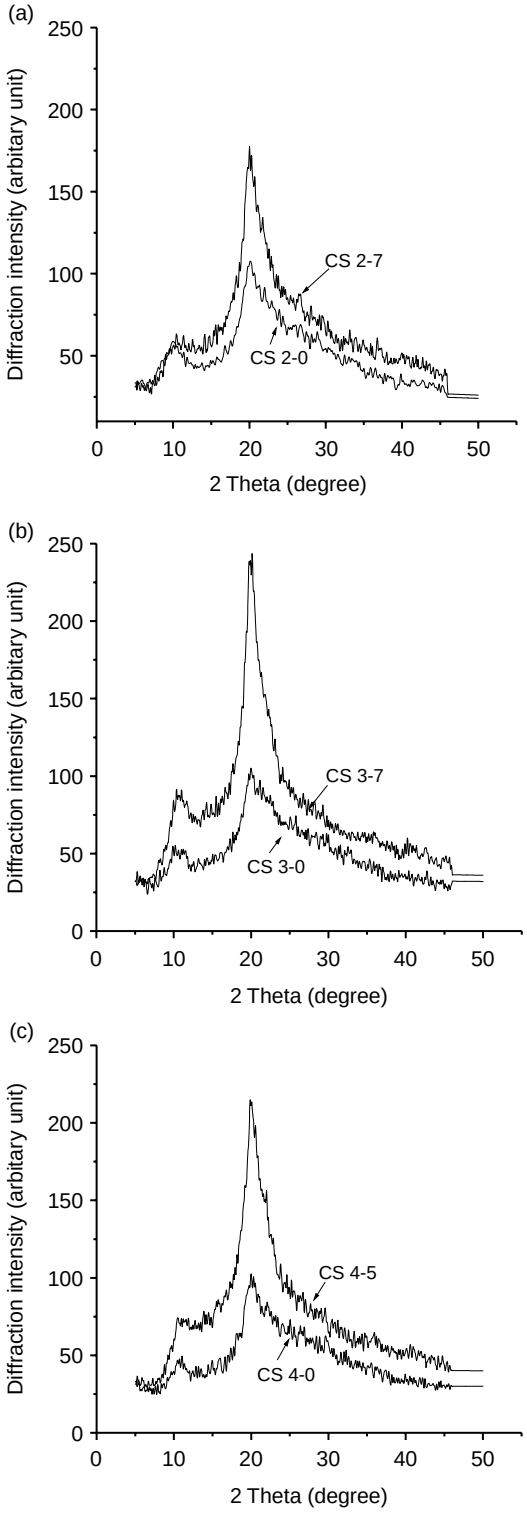

Fig. 7. X-ray diffraction patterns of initial chitosans CS2-0 (a), CS3-0 (b), CS4-0 (c) and their ultrasonic treated products.

3.4. Effect of ultrasonic treatment on the physical structure of chitosan

The wide-angle X-ray diffraction patterns of all the six chitosans (Fig. 7) show their characteristic peaks at $2\theta = 10.4$ and 20.2°, which coincided with the pattern of the 'L−2 polymorph' of chitosan reported previously (Saitô & Tabeta, 1987). The crystallinity of ultrasonic treated chitosans products (CS 2-7, CS 3-7 and CS 4-5) are remarkably higher than those of the initial chitosans. The rise in crystallinity also happened in enzymatic depolymerization (Qin et al., 2004). The present results indicate that ultrasonic treatment changed the physical structure of chitosan, mainly due to the decrease of molecular weight.

3.5. Antimicrobial activity of ultrasonically degraded chitosans with different molecular weights and deacetylation degrees

The curves for the antimicrobial activity of initial and ultrasonic degraded chitosans with different molecular weights and DDs (Fig. 8) suggest that the inhibition rates against *E. coli* and *S. aureus* increase with the decrease of molecular weight caused by ultrasonic degradation. Though the DD of CS 1-0, CS 2-0 and CS 3-0 decreased with depolymerization time, the antimicrobial activity of their degraded products increased rapidly. So for the ultrasonic degraded chitosans, molecular weight is a more important factor than DD in terms of effect on antimicrobial activity.

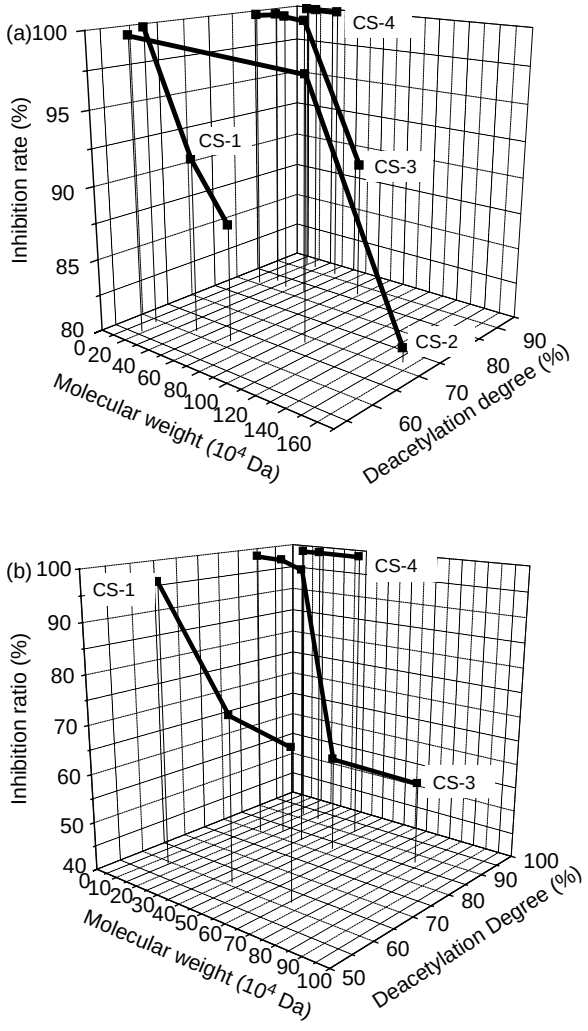

Fig. 8. Effect of Mw and DD of ultrasonic treated chitosas on the inhibition rate against (a) *E. coli*, (b) *S. aureus*.

The effects of molecular weight and DD of chitosan on the antimicrobial activity have been studied by many researchers (Kittur, Vishu Kumar, & Tharanathan, 2003; No et al., 2002; Jeon et al., 2001; Rhoades & Roller, 2000). However, the results are inconsistent, which may be caused by differences in the range of molecular weight, DD, solvent, and microorganism species and sources. The source of the tested chitosan may also be an important factor for the antimicrobial activity. For the chitosan samples from enzymatic and oxidative–reductive degradation, the antimicrobial activity decreased with decreasing molecular weight (No et al., 2002; Jeon et al., 2001; Rhoades & Roller, 2000). However, for those from ultrasonic degradation in the present experiment, the case is contrary. For that from alkaline hydrolysis of chitin, the results are very variable. This may be because the mechanisms of degradation are different for different degradation method, and then the chemical structure and chain conformation of the resultant chitosans are different.

Acknowledgements

We are grateful for the financial support of this research from the National Natural Science Foundation of China (Grant No. 29977014) and the foundation for the Research of Excellent Young Teacher in China University of Geosciences.

References

Begona, C. G., & Ruth, D. (1997). Evaluation of the biological properties soluble chitosan and chitosan microspheres. *International Journal of Pharmaceutics*, *148*, 231–240.

Chen, R. H., Chang, J. R., & Shyur, J. S. (1997). Effects of ultrasonic conditions and storage in acidic solutions on changes in molecular weight and polydispersity of treated chitosan. *Carbohydrate Research*, *299*, 287–294.

Chen, R. H., & Hwa, H. D. (1996). Effect of molecular weight of chitosan with the same degree of deacetylation on the thermal, mechanical, and permeability properties of the prepared membrane. *Carbohydrate Polymers*, *29*, 353–358.

Chen, R. H., & Tsaih, M. L. (1998). Effect of temperature on the intrinsic viscosity and conformation of chitosans in dilute HCl solution. *International Journal of Biological Macromolecules*, *23*, 135–141.

Choi, B. K., Kim, K. Y., Yoo, Y. J., Oh, S. J., Choi, J. H., & Kim, C. Y. (2001). In vitro antimicrobial activity of a chitooligosaccharide mixture against *Actinobacillus actinomycetemcomitans* and *Streptococcus mutans*. *International Journal of Antimicrobial Agents*, *18*, 553–557.

Choi, W. S., Ahn, K. J., Lee, D. W., Byun, M. W., & Park, H. J. (2002). Preparation of chitosan oligomers by irradiation. *Polymer Degradation and Stability*, *78*, 533–538.

Cravotto, G., Tagliapietra, S., Robaldo, B., & Trotta, M. (2005). Chemical modification of chitosan under high-intensity ultrosound. *Ultrasonics Sonochemistry*, *12*, 95–98.

Czechowska-Biskup, R., Rokita, B., Lotfy, S., Ulanski, P., & Janusz, M. (2005). Degradation of chitosan and starch by 360-kHz ultrasound. *Carbohydrate Polymers*, *60*, 175–184.

Dodane, V., & Vilivalam, V. D. (1998). Pharmaceutical applications of chitosan. *Pharmaceutical Science and Technology Today*, *1*, 246–253.

Jeon, Y. J., Park, P. J., & Kim, S. K. (2001). Antimicrobial effect of chitooligosaccharides produced by bioreactor. *Carbohydrate Polymers*, *44*, 71–76.

Kittur, F. S., Vishu Kumar, A. B., & Tharanathan, R. N. (2003). Low molecular weight chitosans—Preparation by depolymerization with *Aspergillus niger* pectinase and characterization. *Carbohydrate Research*, *338*, 1283–1290.

Kondo, Y., Nakatani, A., Hayashi, K., & Ito, M. (2000). Low molecular weight chitosan prevents the progression of low dose streptozotocin-induced slowly progressive diabetes mellitus in mice. *Biological and Pharmaceutical Bulletin*, *23*, 1458–1464.

Kumar, M. N. V. R. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers*, *46*, 1–27.

Li, J., Du, Y., Yang, J., Feng, T., Li, A., & Chen, P. (2005). Preparation and characterization of low molecular weight chitosan and chito-oligomers by a commercial enzyme. *Polymer Degradation and Stability*, *87*, 441–448.

Lim, L. Y., Khor, E., & Koo, O. (1998). Irradition of chitosan. *Journal of Biomedical Materials Research*, *43*, 282–290.

Muzzarelli, R. A. A., & Rocchetti, R. (1985). Determination of the degree of acetylation of chitosans by first derivative ultraviolet spectraphotometry. *Carbohydrate Polymers*, *5*, 461–472.

No, H. K., Park, N. Y., Lee, S. H., & Meyers, S. P. (2002). Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *International Journal of Food Microbiology*, *74*, 65–72.

Qin, C. Q., Du, Y. M., & Xiao, L. (2002a). Effect of hydrogen peroxide treatment on the molecular weight and structure of chitosan. *Polymer Degradation and Stability*, *76*, 211–218.

Qin, C. Q., Du, Y. M., Xiao, L., Li, Z., & Gao, X. H. (2002b). Enzymic preparation of water-soluble chitosan and their antitumour activity. *International Journal of Biological Macromolecules*, *31*, 111–117.

Qin, C. Q., Du, Y. M., Zong, L. T., Zeng, F. G., Liu, Y., & Zhou, B. (2003). Effect of hemicellulase on the molecular weight and structure of chitosan. *Polymer Degradation and Stability*, *80*, 435–441.

Qin, C. Q., Zhou, B., Zeng, L. T., Zhang, Z. H., Liu, Y., Du, Y. M., et al. (2004). The physiochemical properties and antitumor activity of cellulase-treated chitosan. *Food Chemistry*, *84*, 107–115.

Rhoades, J., & Roller, S. (2000). Antimicrobial actions of degraded and native chitosan against spoilage organisms and laboratory media and foods. *Applied and Environmental Microbiology*, *66*, 80–86.

Richardson, S. C. W., Kolbe, H. V. J., & Duncan, R. (1999). Potentials of low molecular mass chitosan as a DNA delivery system, biocompatibility, body distribution and ability to complex and protect DNA. *International Journal of Pharmaceutics*, *178*, 231–243.

Roller, S., & Covill, N. (1999). The antifungal properties of chitosan in laboratory media and apple juice. *International Journal of Food Microbiology*, *47*, 67–77.

Saitô, H., & Tabeta, R. (1987). High-resolution solid-state ^{13}C-NMR study of chitosan and its salts with acids. *Macromolecules*, *20*, 2424–2430.

Seo, W. G., Pae, H. O., & Chung, H. T. (2000). Synergistic cooperation between water-soluble chitosan oligomers and interferon-γ for induction of nitric oxide synthesis and tumoricidal activity in murine peritoneal macrophages. *Cancer Letters*, *159*, 189–195.

Shahidi, F., Arachchi, J. K. V., & Jeon, Y. J. (1999). Food applications of chitin and chitosans. *Trends in Food Science and Technology*, *10*, 37–51.

Sugano, M., Yoshida, K., Hashimoto, M., Enomoto, K., & Hirano, S. (1992). Hypocholesterolemic activity of partially hydrolyzed chitosan in rats. In C. J. Brine, P. A. Sandford, & J. P. Zikakis (Eds.), *Advances in chitin and chitosan* (pp. 472–478). London: Elsevier.

Suzuki, K., Mikami, T., Okawa, Y., Tokoro, A., Suzuki, S., & Suzuki, M. (1986). Antitumor effect of hexa-*N*-acetylchitohexaose and chitohexaose. *Carbohydrate Research*, *151*, 403–408.

Trzciński, S., & Staszewska, D. U. (2004). Kinetics of ultrasonic degradation and polymerization degree distribution of sonochemically degraded chitosans. *Carbohydrate Polymers*, *56*, 489–498.

Tsaih, M. L., & Chen, R. H. (1997). Effect of molecular weight and urea on the conformation of chitosan molecules in dilute solutions. *International Journal of Biological Macromolecules*, *20*, 233–240.

Tsaih, M. L., & Chen, R. H. (2003). Effect of degree of deacetylation of chitosan on the kinetics of ultrasonic degradation of chitosan. *Journal of Applied Polymer Science*, *90*, 3526–3531.

Vishu Kumar, A. B., Varadaraj, M. C., Lalitha, R. G., & Tharanathan, R. N. (2004). Low molecular weight of chitosans: Preparation with the aid of papain and characterization. *Biochimica et Biophysica Acta*, *1670*, 137–146.

Wang, W., Bo, S., Li, S., & Qin, W. (1991). Determination of the Mark-Houwink equation for chitosans with different degrees of deacetylation. *International Journal of Biological Macromolecules*, *13*, 281–285.

Wang, W., & Qin, W. (1989). Ultrasonic degradation of deacetylated chitin. *Huaxue Tongbao*, *9*, 41–44.

Zheng, L. Y., & Zhu, J. F. (2003). Study on antimicrobial activity of chitosan with different molecular weights. *Carbohydrate Polymers*, *54*, 527–530.