Moisture Retention and Antibacterial Activity of Modified Chitosan by Hydrogen Peroxide

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ABSTRACT: A series of chitosans with various molecular weights from 1.2×10^3 to 30.0×10^4 were prepared by oxidative degradation with H_2O_2 and characterized by IR, ^{13}C-NMR, and gel permeation chromatography. Their carboxylic contents increased with a decrease in molecular weight (M_w). The moisture-absorption and moisture-retention capacities of resulting chitosans were dependent on both the molecular weight and the degree of deacetylation (DD). Microcalorimetry was first used to study the kinetics of action of the chitosans on a strain of *Staphylococcus aureus* at pH 7. The antibacterial activity of the water-soluble chitosan against *S. aureus*, *Escherichia coli*, and *Salmonella typhi* was evaluated by the conventional agar plate method at pH 7. The water-soluble product with M_w of 0.45×10^4 from initial chitosan of DD of 90% showed high moisture-absorption and moisture-retention capacities, and <2% concentration can completely inhibit the growth of these bacteria. © 2001 Wiley Periodicals, Inc. J Appl Polym Sci 86: 1724–1730, 2002

Key words: degradation; polysaccharide; renewable resource; structure-property relation

INTRODUCTION

Chitosan, the deacetylated derivative of chitin, is an abundant, renewable, nontoxic, and biodegradable carbohydrate polymer and is available largely in the exoskeletons of shellfish and insects. Therefore, chitosan has received much attention as a functional biopolymer for diverse applications, especially in pharmaceutics,[1] food,[2] and cosmetics.[3] These functions are dependent not only upon their chemical structure but also on the molecular size. For example, the carboxymethylation of chitosan imparts better moisturizing properties[4]; low-molecular-weight (LMW) chitosans are known to have biological activities such as medicinal actions to living bodies and antibacterial activities.

The LMW chitosan can be prepared by enzymic and chemical degradation of the polymer chain. Enzymic hydrolyses involve mild process conditions and lead to the production of chitooligosaccharide (COS) with relatively high yields.[5,6] However, they increase the average cost of production because the enzymes are too expensive to be commercialized. In addition, the products contain chitosan pyrogenicity caused by the presence of proteins of the enzyme complex. Various acids such as hydrochloride and phosphoric acid have always been used for hydrolysis of chitosan to obtain LMW chitosans,[7,8] but it seemed to be difficult to obtain chitosan with a preselected size and the downstream procedure is tedious. Many oxidizing agents are also being used for the degradations,[9,10] but most of them are toxic reagents such as chrome compounds[11] that are not desirable for application in cosmetics, pharmaceutics, and food. Nevertheless, hydrogen peroxide has long been used to the degradation of polysaccharides[12] because the method possessed the following advantages: production of chitosans of a preselected size, low cost of production, simple work-up procedure, and capability of being a nontoxic reagent. Therefore, we desired to investigate the functional properties of these modified chitosans by H_2O_2. An understanding of the effects of chemical structure and molecular weight on the functional properties is essential to the successful application of chitosan. It is worth noting that the advanced cosmetics ask for high moisture-retention capacity and antibacterial activity at wider pH ranges, especially at pH > 6. In this article, the moisturizing properties and antibacterial activity of resultant chitosans degraded by using H_2O_2 were studied.

EXPERIMENTAL

Materials

Chitosans were purchased from Yuhuan Biochemical Co., Zhejiang, China. Their weight-average molecular weights were determined by capillary viscometry[13] and their degrees of deacetylation (DD) were deter-

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mined with potentiometric titration method[14] (M_v = 78.1 × 10^4 and DD = 90.5% for Ch90; M_v = 82.0 × $10^4$and DD = 85.5% for Ch85; M_v = 80.2 × 10^4 and DD = 92.2% for Ch92; M_v = 38.5 × 10^4 and DD = 95.6% for Ch95; M_v = 127.0 × 10^4 and DD = 73.2% for Ch73). Hyaluronic acid (HA) with M_w = 126 × 10^4 was the USA commercial product. *N*-acetylglucosamine (NAG) was the product of TCI, Japan. COS was prepared from hydrolysis of Ch90 with cellulase.[15] *O*-carboxymethylated chitosan (CMCH) was obtained from the Ch90 by the method described previously.[4] All other chemicals were of reagent grade and were supplied by Beijing Shiji Co. (Beijing, China) and Shanghai Chemical Co. (Shanghai, China).

Degradation of chitosan

Chitosan powder (10 g) was added into a 1000-mL reactor with 500 mL of deionized water and stirred at 65°C for 1 h. Then, 5 mL 30% hydrogen peroxide solution was added. At the end of the predetermined time, the mixture was then subjected to vacuum filtration. The solid was then washed with deionized water and finally collected by drying at 30°C. The filtrate was concentrated to 30 mL under reduced pressure at 50°C, and a precipitate was obtained by addition of 200 mL ethanol to the mixture. The precipitate was washed with ethanol, and the water-soluble chitosan was collected after drying over phosphorus pentoxide in vacuum.

The resulting chitosan was reprecipitated with acetone from water solutions at pH 1 and 12 and then washed with ethanol to obtain the chitosan hydrochlorides and sodium salts, respectively.

Characterization

Weight-average molecular weight (M_w), number-average molecular weight (M_n), and molecular weight dispersion (M_w/M_n) were measured by GPC. The GPC equipment consisted of the connected column (TSK G5000-PW and TSK G3000-PW), TSP P100 pump, and RI 150 refractive index detector. The flow rate was maintained at 1.0 mL min^{-1}. An amount of 0.2 mol L^{-1} CH_3COOH/0.1 mol L^{-1} CH_3COONa was used as the eluant. 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.

^{13}C-NMR spectra were recorded on a Varian Mercury V×300 spectrometer. Water-soluble samples were dissolved in D_2O and water-insoluble samples were dissolved in D_2O in the presence of CD_3COOD.

IR spectra were taken with KBr pellets on a Shimadzu FTIR-8210 spectrophotometer.

Determination of content of carboxylic group

The resultant chitosans were treated with 1% ammonia in ethanol, and the precipitates were thoroughly washed with ethanol. The chitosans were collected after drying in vacuum. The dried sample of chitosan (0.2 g) was dissolved in V_{B1} ml (1–5 mL) of 0.1 mol L^{-1} NaOH and evaporated to dryness under vacuum at 40°C. The mixture was dissolved in 10.0 mL of 0.1 mol L^{-1} HCl. The solution was then titrated with 0.1 mol L^{-1} NaOH and the volume of consumed NaOH V_{B2} mL was determined[14] by the titration curve recorded on a Delta-320-S pH meter. The content of carboxylic group of sample was calculated by $X = (V_B N_B - 10.0 N_A)/W$, where N_A is the HCl concentration; $V_B = V_{B1} + V_{B2}$; N_B is the NaOH concentration, and W is the sample weight.

Estimation of water solubility

An amount of 0.5 g chitosan was dissolved in 20 mL 0.1 mol L^{-1} HCl and was then neutralized with 1 mol L^{-1} NaOH. The pH values were recorded on a Delta-320-S pH meter when cloudy precipitate began to appear. Ten portions (2 mL/each) taken out from the mixtures at different pH values near the cloud point were left in the test glasses for 24 h. The pH value at which the precipitation started was examined and recorded.

Moisture-absorption and moisture-retention capacity test

The powdered chitosans were the fraction that passed through a 140-mesh sieve. The sample powders in weighing bottles were further dried over P_2O_5 at 40°C for 24 h in vacuum before test. The test was carried out according to the method employed by Shuichi et al.[16] The moisture-absorption capacity of the sample was evaluated by the percentage of weight increase of dry sample: R_a (%) = 100 × $(W_n - W_0)/W_0$, where W_0 and W_n were the weight of sample before and after being placed in desiccator where the humidity was controlled by saturated $(NH_4)_2SO_4$ (81% relative humidity) and saturated K_2CO_3 solutions (43% relative humidity), respectively, at 20°C for 40 h. The water-retention capacity of the sample was indicated by the percentage of residual water in the wet sample (wet samples were prepared by adding 10% of water to dry samples): R_h(%) = 100 × H_n/H_0, where H_0 and H_n were the water weight of sample before and after being placed in the silica-gel desiccator at 20°C for 40 h.

Antibacterial assessment with microcalorimetry

Staphylococcus aureus was provided by China Center for Type Culture Collection, Wuhan University. The

TABLE I
Molecular Weight and Solubility of Degraded Chitosan

Sample code	$M_w \times 10^{-4}$	M_w/M_n	pH of precipitation
CS0	30	5.37	6.1
CS1	4.8	2.30	6.5
CS3	1.2	1.98	6.6
CS4	0.77	1.77	7.0
CL5	0.45	1.58	—
CL6	0.32	1.27	—
CL7	0.12	1.16	—
CL95	0.47	1.61	—
CL92	0.45	1.48	—
CL85	0.41	1.52	—
CL73	0.39	1.62	—

peptone culture medium contained 1000 mL (pH = 7.2): NaCl 5 g, peptone 5 g, beef extract 5 g. It was sterilized in high-pressure steam at 120°C for 0.5 h. Tested chitosans were prepared from initial chitosan of DD 90.5%.

A microcalorimeter, LKB-2277 Bioactivity Monitor, manufactured by LKB Corp., Sweden was used to obtain the metabolic power-time curves of the bacteria. The microcalorimeter was thermostated at 37.0°C. The voltage signal was recorded by an LKB-2210 recorder. The method was used in this experiment as previously described.[17]

The power-time curves obtained when a culture of the test bacteria was inoculated with chitosans at 1.0–5.0 g L^{-1}, obey the equation:

$$\ln P_t = kt + \ln P_0$$

Using the equation, the multiplication rate k of these experiments were calculated and the generation times G, which equal (ln 2)/k, were also obtained.

Antibacterial assessment *in vitro* by agar plate method

Test cells were *S. aureus, Escherichia coli,* and *Salmonella typhi* from Hubei Geriatrics Institute, Wuhan, China. The agar plate method was used to determine the minimum inhibition concentration (MIC) of modified chitosans as follows.

A loopful of each culture was spread to give single colonies on nutrient broth (beef extract 3 g, peptone10 g, NaCl 5 g to 1000 mL distilled water, pH 7.0) and incubated at 37°C for 18 h. A representative colony was picked off with a wire loop and placed in a nutrient agar (agar 10 g, beef extract 3 g, peptone10 g, NaCl 5 g to 1000 mL distilled water, pH 7.0), which was then incubated at 37°C for 12 h. By appropriately diluting with sterilized distilled water, each culture containing about 10^7 cells/mL was prepared, which was used for the antibacterial test. The tested chitosan was dissolved in sterile distilled water to the desired concentration. Exposure of the bacterial cells to the antibacterial agent started when the bacterial culture of 18.0 mL was added to antibacterial agent solution of 2.0 mL, which was preequilibrated at 40°C. After inoculation, the plates were incubated at 37°C for 24 h, and the colonies were counted and the MICs were obtained.

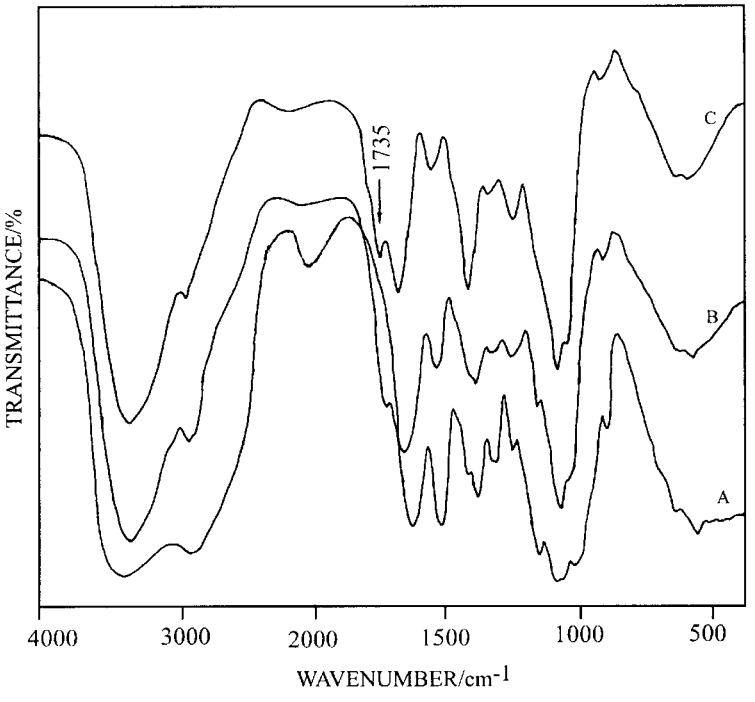

Figure 1 FTIR spectra of chitosan hydrochlorides of (A) Ch90, (B) CL5, and (C) CL7.

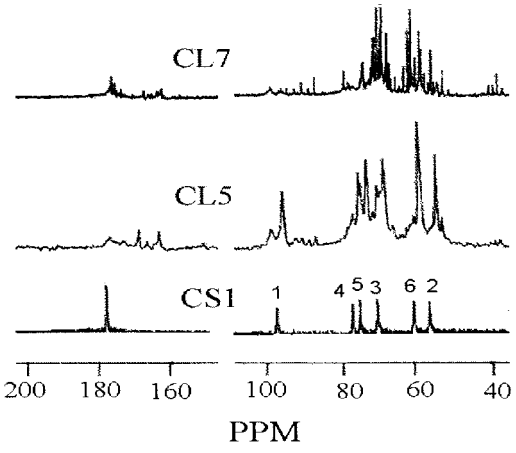

Figure 2 ^{13}C-NMR spectra of sample of CS1, CL5, and CL7.

RESULTS AND DISCUSSION

Characterization of degraded chitosan

A series of samples, depolymerized by H_2O_2 at 65°C for 0–48 h, were studied. The calculated values of M_w and M_w/M_n obtained by GPC are given in Table I. The samples CS0 to CS4, CL5 to CL7 came from the degradation of Ch90, and samples CL95, CL92, CL85, and CL73 were prepared from Ch95, Ch92, Ch85, and Ch73, respectively. The M_w of the product decreased with the prolonged reaction time. Figure 1 showed the IR spectra of the hydrochloride salts of Ch90 and its degraded products. One perceptible difference of the IR spectra of the resultant chitosan hydrochloride from the initial Ch90 hydrochloride was an absorption band at 1735 cm^{-1}, which was assigned to the carboxylic group. With decreasing of M_w of resultant chitosan, the 1735 cm^{-1} band of the hydrochloride became more evident, suggesting that the carboxylic group increased. The ^{13}C-NMR spectra of resultant chitosan CL5 and its sodium salt also gave the signals of carboxylic groups[18] in downfield shift from165 to 180 ppm. The ^{13}C-NMR spectra of resultant chitosan CS1, CL5, and CL7 were shown in Figure 2. The data in Figure 3 also indicated that the content of carboxylic group increased with the decrease of the M_w of resultant chitosan.

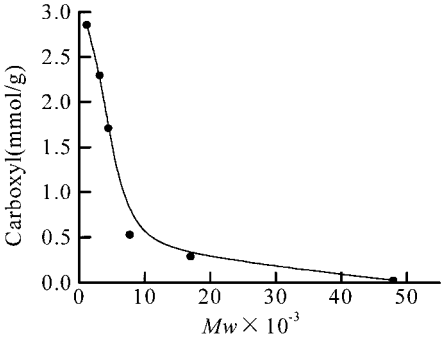

Figure 3 Plot of carboxyl content versus M_w of degraded chitosan.

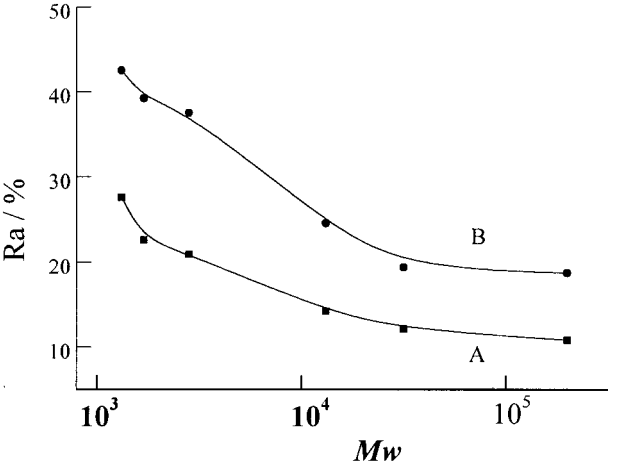

Figure 4 Plot of moisture–absorption capacity versus the molecular weight of resulting chitosan at 43% (A) and 81% (B) relative humidity.

Solubility of degraded chitosan

Water solubility of the chitosan was evaluated in terms of pH values at which the precipitation started from its acidic solution. As shown in Table I, lowering the molecular weight helps to improve solubility.[19] The water solubility was higher as the chitosan molecule was depolymerized to a smaller molecule. The value of pH, at which the chitosan was completely soluble, was smaller as the M_w was higher.[20] When the molecular weight was smaller than $5 \times 10,^3$ the chitosan in water formed clear aqueous solutions and was water-soluble, suggesting that its intramolecular and intermolecular hydrogen bonding in chitosan decreased. In addition, the increased number of carboxylic group also contributed a lot to the improved water solubility because the M_w of water-soluble chitosans, which were prepared by enzymic hydrolysis of Ch90, were generally lower than 2×10^3 (DP $<$ 12) from the results of GPC data.

Moisture-absorption and moisture-retention capacity

Figure 4 shows the relationship between the moisture-absorption capacity and the M_w of resultant chitosan prepared from oxidative degradation of Ch90. The moisture-absorption capacity of chitosan increased with the decrease of M_w, and the increase was rapid when M_w was from1.0×10^4 to 0.4×10^4. With the decrease of M_w, the number of newly produced absorption sites (chain end group and carboxylic group) per unit weight increased and the total amount of absorbed water increased.

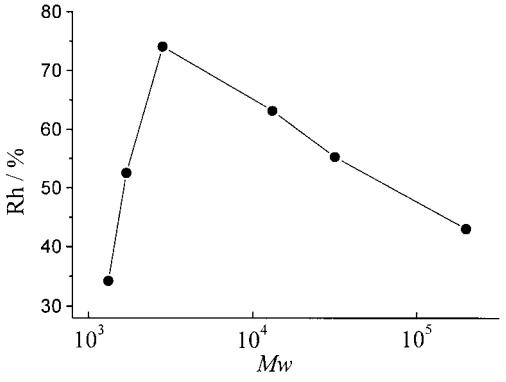

Figure 5 Moisture-retention capacity versus the molecular weight of resultant chitosan in silica gel desiccator.

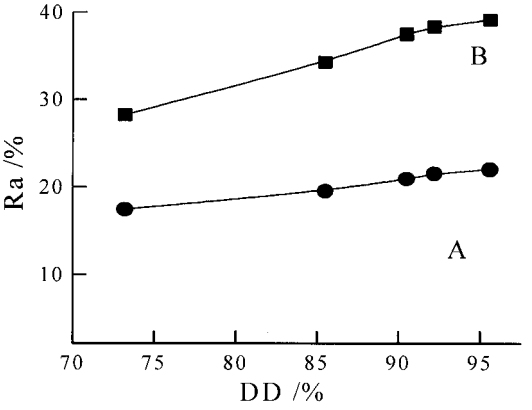

Figure 6 Dependence of moisture-absorption capacity on DD of starting chitosan at 43% (A) and 81% (B) relative humidity.

The results of moisture retention of the resultant chitosan versus the molecular weights are depicted in Figure 5. With the decrease of M_w, the moisture-retention capacity in silica gel first increased and then declined. The optimum M_w for maximum moisture-retention capacity was about 0.45×10^4. The water-soluble chitosan with higher M_w exhibited better moisture-retention effects. One important factor might be the preservation of the space net structure in chitosan, and the other was the decrease of M_w in combination with the proper increase of carboxylic group content. When M_w is smaller than 0.3×10^4, the chemical structure was destroyed because of the increase of partial ring-open oxidation. As shown in Figure 2, not only the signals in downfield increased, but also several signals appeared in the upfield shift at around 40 ppm in the ^{13}C-NMR spectrum of CL7. It is certain that the molecular structure of CL7 was severely destroyed. Simultaneously, the space net structure was also destroyed after the chitosan was degraded to COS.[7,8] Therefore, although the moisture-absorption increased, the water-holding capacity decreased. The results suggest that the proper chain length can be regarded as one of the essential elements for chitosan to moisture retention. As shown in Table II, the COS prepared by enzymic hydrolysis exhibited excellent moisture-absorption and moisture-retention capacity, but for NAG, one monosaccharide had little.

The amino content in chitosan was directly related to this functional property. Both Ch90 and chitin had high M_w, but the Ch90 had better moisture-absorption and moisture-retention capacity than chitin because of the higher amino content of Ch90. For the degraded chitosan, the degree of deacetylation of starting chitosan had a similar influence. The chitosans with M_w of 0.45×10^4 were water-soluble and had better moisture absorption and moisture retention as described above, so they were selected for further investigation. Figures 6 and 7 show the effect of DD of initial chitosan on the moisture-absorption and moisture-retention capacities of water-soluble chitosan, respectively. With an increase in amino group content, there was an increase in moisture-absorption and moisture-retention capacities. The chitosan prepared from initial chitosan with DD > 90% achieved higher moisture-retention capacity.

Table II shows the moisture-absorption and moisture-retention capacities of the chitosan, its hydrochloride, and sodium salt in the order of CL5 > CL5-Na > CL5-HCl. CL5 and its sodium salt exhibited almost

TABLE II
Moisture-Absorption and Moisture-Retention Capacities

Samples	R_a (%) of dried samples		R_h (%) of wet samples
	A[a]	B[a]	C[a]
CL5	20.9	37.5	74.0
CL5-HCl	13.6	28.5	56.5
CL5-Na	18.6	33.9	72.1
HA	14.8	36.7	58.3
COS	21.2	34.1	78.3
Ch90	10.8	18.7	34.2
Chitin	7.5	12.8	24.5
NAG	0.2	0.5	1.8

[a] A, 43% relative humidity; B, 81% relative humidity; C, in silica-gel desiccator.

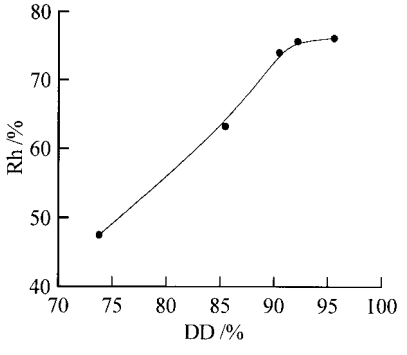

Figure 7 Dependence of moisture retention on DD of starting chitosan in silica-gel desiccator.

TABLE III
Rate Constant (k) and Generation Time (G) of *S. aureus*

Sample	Conc. (g L^{-1})	k(min^{-1})	G(min)	R^*
Control	—	0.0227 ± 0.0008	30.5	0.9991
CS3-HCl	1.0	0.0115	60.2	0.9903
	2.0	0.0000		
CL5-HCl	2.0	0.0193	37.1	0.9959
CL5	1.0	0.0179	38.6	0.9902
	2.0	0.0147	47.2	0.9909
	5.0	0.0099	70.2	0.9990
CL5-Na	1.0	0.0142	48.8	0.9936
	2.0	0.0000	—	—
CMCH	2.0	0.0219	31.6	0.9982

R, correlation coefficient.

the same excellent moisturizing property as HA, one of the typical advanced but expensive moisture-retention materials. The result indicated that the application of the chitosan was promising in the media of pH > 6.

Antibacterial activities

Chitosan confers considerable antimicrobial activity against a broad spectrum of microorganisms.[2] The antimicrobial activities, which were not discovered in chitin, mainly depended on the type of functional groups in chitosan and the molecular weight of the base chitosan. However, the reported antimicrobial activities of chitosans were generated in aqueous acidic environment, although they had little antimicrobial activities at pH 7.0.[21] The aim of this research was therefore to investigate the antibacterial activity of modified chitosan at media of pH 7.0–7.2 for the potential use.

Table III shows the data from the power-time curves by the microcalorimetry. The time of the log phase that is between the start of the experiment and the ascending phase of the power suggested that retarding times of bacteria growth were longer and the generation time G increases with an increase in the concentration of chitosan. It might be that some of the bacteria are killed by the chitosan so that it took longer to generate a detectable signal. The experiment indicated that the chitosans all have the ability to inhibit the metabolic growth of *S. aureus* to different extents.

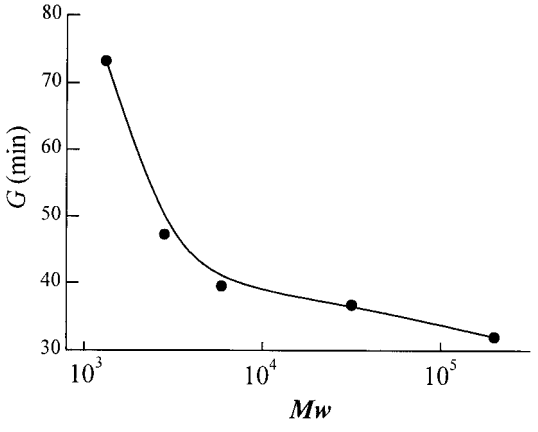

Figure 8 Generation time G versus the molecular weights of degraded chitosan.

Figure 8 demonstrates that the antibacterial activity of oxidized chitosan was obviously affected by the molecular weights. The generation time increased with a decrease in M_w of chitosan at the same concentration. The oxidized chitosan with lower M_w gave better inhibitory effect on *S. aureus.* One of the reasons may be their solubility above pH > 6.5. This result was supported by the agar plate method in Table IV.

The chitosan hydrochlorides, which were water-soluble, had high antibacterial activities, as reported by many articles,[2] due to the positively charged cations. The higher M_w chitosan hydrochloride CS3-HCl showed higher antibacterial activity than CL5-HCl, which might be because the oxidized chitosan of lower M_w lost more amino group (confirmed by elemental analysis data and IR) in the degradation process, so it formed less polycations.

In this experiment, it is interesting that the inhibitory effect is in the order of CL5-Na > CL5 > CL5-HCl. At the concentration of 5 g/L, sodium salt of CL5 inhibited the metabolism of *S. aureus* completely. In nonacidic media, the antibacterial action of resultant chitosan might be that chitosan acted as a chelating agent rendering metals, trace elements, or essential

TABLE IV
MIC of Resultant Chitosan (g/L)

Sample	*S. aureus*	*E. coli*	*Sa. typhi*
CL5	5.0	20	20
CL6	2.5	10	10
CL8	1.25	5.0	2.5
CS4-HCl	0.32	1.25	1.25
CS2-HCl	0.16	1.25	1.25
COS	>20.0	40.0	40.0
CMCH	>40.0	>40.0	>40.0

nutrients unavailable for the organism to grow at a normal rate.[22,23] The resultant water-soluble chitosan possessed both —NH_2 and —COO^-, which were in the proper positions to form chelate ring. Therefore, the antibacterial action of chitosan might be caused by comprehensive factors. In acidic media, the polycationic nature played a main effect: in weak basic media, chelate effect might be the most important factor; in neutral media, the combined factors imparted the function.

Table IV listed minimum inhibition concentration of the resulting chitosans. The antibacterial tests were conducted using water-soluble chitosans (prepared from Ch90) at pH 7.0–7.2. The resultant chitosan with a low M_w and high carboxylic content had low MIC and showed high antibacterial activity. This is in good agreement with the above result.

CONCLUSION

Chitosan could be degraded by using hydroperoxide to produce the low-molecular-weight chitosans with M_w from 3.0×10^5 to1.2×10^3. FTIR and ^{13}C-NMR confirmed that the carboxylic group was introduced onto degraded chitosan and the carboxylic content increased with the decrease of M_w. The functional properties of chitosan were dependent on the molecular weight and the degree of deacetylation. The moisture-retention capacity first increased with the decreasing M_w up to 0.45×10^4 and then decreased. The antibacterial activity of the degraded chitosan at pH 7 media increased with the decrease of M_w. The high DD enhanced these properties of chitosan. The water-soluble chitosan with high molecular weight and high DD had not only good water-solubility and moisture-retention capacity, but also possessed broader spectra of antibacterial activity even at pH $>$ 6.5. Thus, they have promising applications in advanced cosmetics, medicinal aids, food, and other fields.

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