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Analytical Methods

An innovative method for preparation of acid-free-water-soluble low-molecular-weight chitosan (AFWSLMWC)

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Abstract

The ozone generated from compressed oxygen by a laboratory-scale corona discharge generator was used for the preparation of acid-free-water-soluble low-molecular-weight chitosan (AFWSLMWC). Factors affecting the percent yield of AFWSLMWC were studied in batch experiments. AFWSLMWC with a molecular weight of 4.3–13.1 kDa was obtained. IR spectra demonstrated that the chemical structures of AFWSLMWC were not modified during the depolymerisation process. There was no significant change of the total degree of deacetylation (DD) of AFWSLMWC, compared with the initial chitosan. The method is promisingly suitable for scale-up manufacture of acid-free-water-soluble low-molecular-weight chitosan.

Keywords: Acid-free-water-soluble; Low-molecular-weight chitosan; Ozone; Percent yield; Chitosan; Weight-average molecular weight

1. Introduction

Chitosan, the deacetylated derivative of chitin, is one of the abundant, renewable, nontoxic and biodegradable carbohydrate polymers, and available largely in the exoskeletons of shellfish and insects. Therefore, chitosan has received much attention as a functional biopolymer for diverse applications, especially in food (Shahidi, Arachchi, & Jeon, 1999), pharmaceutics (George & Abraham, 2006), biomaterials (Khor & Lim, 2003; Suh & Matthew, 2000), medicine and cosmetics (Rinaudo, 2006). These functions have been shown to be dependent not only upon the chemical structure of chitosan but also its molecular size. For example, the application of the native polysaccharide in medicine and the food industry is limited by its high molecular weight, resulting in its low solubility in acid-free aqueous media. Some chitosan applications require as a starting material a low-molecular-weight chitosan with a high solubility, as well as a low viscosity in acid-free water. Thus it is necessary to convert chitosan to an acid-free-water-soluble low-molecular-weight chitosan (AFWSLMWC). Acid-free-water-soluble low-molecular-weight chitosan is known to have many kinds of biological activities, such as antifungal activity (Chien, Sheu, & Lin, 2007; Tikhonov, Stepnova, Babak, & Yamskov, 2006), antimicrobial activity (Liu et al., 2006), and antitumour activity (Qin et al., 2004).

AFWSLMWC can be prepared by enzymatic and chemical degradation of the chitosan polymer (Biskup, Rokita, Ulanski, & Rosiak, 2005; Huang, Wang, Huang, Zhuo, & Guo, 2007; Jiao & Shen, 2002; Kang, Dai, Zhang, & Chen, 2007; Li et al., 2005; Qin, Du, & Xiao, 2002; Qin et al., 2003; Wang, Huang, & Wang, 2005; Wasikiewicz, Yoshii, Nagasawa, Wach, & Mitomo, 2005). The chemical approach has several defects: harsh conditions of hydrolysis, low yields of the product, chemical modifications of glucose ring and others. Especially, the downstream

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procedure is tedious. The enzymatic process for AFWSLMWC preparation seems to be generally preferable to chemical reactions because enzymes can and do operate under non-ideal conditions and are highly specific. Furthermore, the enzymatic method also minimises alteration in the chemical nature of the product. For commercial utilisation, however, the prohibitive cost of the specific and non-specific enzymes such as chitosanase, chitinase and hemicellulase inhibits their use on an industrial scale.

Our research group recently obtained acid-free-water-soluble low-molecular-weight chitosan by ozone treatment. Preparation of acid-free-water-soluble low-molecular-weight chitosan by ozone treatment is a new method which has never been reported in the literature. The present paper describes the preparation of acid-free-water-soluble low-molecular-weight chitosan by ozone treatment, which could be an alternative economical way of obtaining AFWSLMWC from chitosan. Factors affecting the percent yield of AFWSLMWC were studied and the relationship between the factors and the percent yield of AFWSLMWC was clarified. The structural identification of AFWSLMWC was performed using an IR method.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Jinke Biochemical Co. (Zhejiang, China). The degree of deacetylation and weight-average molecular weight (M_w) of the commercial chitosan used in this study were determined experimentally.

Degree of deacetylation (DD) was 89.8%, and the weight-average molecular weight (M_w) was 245.9 kDa. Oxygen with a purity of above 99% was purchased from NanNing Gas Station. All other chemicals were of reagent grade.

2.2. Measurement of molecular weight distribution by gel permeation chromatography (GPC)

Weight-average molecular weight (M_w), number average molecular weight (M_n) and molecular weight dispersion (M_w/M_n) of acid-free-water-soluble low-molecular-weight chitosan were measured by GPC. The GPC equipment consisted of a column (TSK G3000-PWXL), TSP P100 pump and RI 150 refractive index detector. The eluent was 0.2 M CH_3COOH/0.1 M CH_3COONa. Eluent and chitosan sample solutions were filtered through 0.45 μm Millipore filters. The flow rate was maintained at 1.0 ml/min. The sample concentration was 0.4 mg/ml. The standards used to calibrate the column were Tosoh pullulan (Tosoh Corporation, Tokyo, Japan). All data provided by the GPC system were collected and analysed using the Jiangshen Workstation (Dalian, China) software package.

Weight-average molecular weight of the initial chitosan was measured using connected columns (TSK G5000-PW and TSK G3000-PW).

2.3. Characterisation of acid-free-water-soluble low-molecular-weight chitosan

Samples of acid-free-water-soluble low-molecular-weight chitosan were dissolved in aqueous HCl; then the sample was reprecipitated with ethanol from the aqueous HCl, and washed with ethanol, to obtain the acid-free-water-soluble low-molecular-weight chitosan hydrochloride salt. The dried hydrochloride samples were used for FT-IR analysis.

Fourier transformed infrared spectroscopy was conducted on an FT-IR spectrometer (Nicolet 5DXB, Nicolet Biomedical Inc., Madison, WI) in the range between 4000 and 400 cm^{-1}, with a resolution of 4 cm^{-1}. Thirty two scans at a resolution of 4 cm^{-1} were averaged and referenced against air. All powder samples were compressed into KBr disks for the FT-IR measurement.

2.4. Potentiometric determination of the degree of deacetylation (DD)

The chitosan (0.1 g) was dissolved in a known excess of 0.1 M HCl acid (10 ml). From the titration of this solution 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 (Tolaimate et al., 2000). The titration was performed with a pH meter.

2.5. Experimental facility and batch experiments for the determination of appropriate reaction conditions

Unless otherwise stated, the preparation of acid-free-water-soluble low-molecular-weight chitosan was carried out as follows: All the experiments were done in a 300 ml Pyrex cell (Fig. 1) in which 200 ml chitosan solution was introduced. The ozone was generated from compressed oxygen with a purity of above 99% by a laboratory-scale corona discharge generator and bubbled into the bottom of the reactor through a thin bubble diffuser. A mixer with a mixing speed of 200 rpm was used to provide good contact between the chitosan solution and ozone. The application rates of ozone can be adjusted by controlling the flow rate of oxygen flowing into the laboratory-scale corona discharge generator. Inlet concentration of O_3 in oxygen was determined by passing the ozone gas through a potassium iodide trap and titrating with standardized sodium thiosulfate.

The aim of this work is the development and optimisation of further possible reactions for the preparation of acid-free-water-soluble low-molecular-weight chitosan. Therefore batch experiments on a laboratory scale are

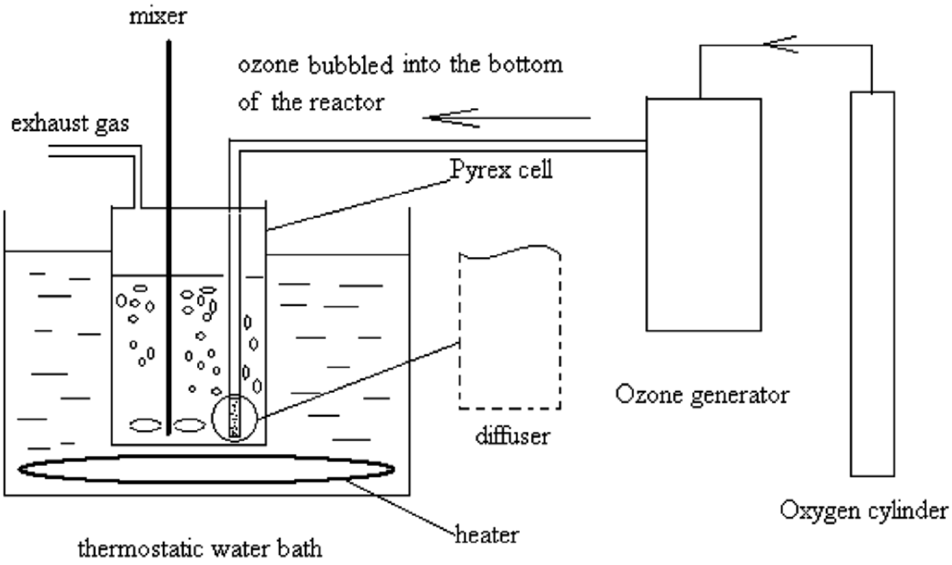

Fig. 1. Sketch of the experimental procedure.

needed to find appropriate reaction conditions. The reaction temperature, the reaction time, and the application rates of ozone were studied in batch experiments. Details of the methods used in batch experiments are given in the following text.

2.6. Preparation of acid-free-water-soluble low-molecular-weight chitosan

Chitosan (10 g) was completely dissolved in 200 ml of 2% (v/v) acetic acid solution. The solution in the reaction vessel was placed in a thermostatic water bath at 50 °C and the generated O_3 was then bubbled into the reactor by means of a diffuser, to initiate reaction. The flow rate of ozone was 65 ± 5 mg/min. After 20 min, ozone treatment was terminated by shutting off the O_2 from the cylinder, and the 200 ml chitosan solution treated with O_3 was removed. The solution was concentrated to about one-twentieth with a rotary evaporator under reduced pressure. The concentrate was neutralised to pH 12 by adding concentrated KOH solution and then precipitated by adding ethanol. The precipitates were washed thoroughly with ethanol. The degraded chitosan ($DC_{20\ min}$) was finally obtained after collecting the precipitate and drying it over phosphorus pentoxide under vacuum. $DC_{40\ min}$, $DC_{60\ min}$, and $DC_{80\ min}$ were obtained using the same method, using ozone treatment times of 40, 60 and 80 min, respectively.

Each degraded chitosan sample was suspended in 200 ml distilled water at 25 °C for 2 h with constant mixing. The filtrate containing acid-free-water-soluble low-molecular-weight chitosan was collected by filtration. The collected filtrate was concentrated to about one-twentieth with a rotary evaporator under reduced pressure. Acid-free-water-soluble low-molecular-weight chitosan in the concentrated solution was precipitated by adding ethanol. The precipitate was washed thoroughly with ethanol. Acid-free-water-soluble low-molecular-weight chitosan (AFWSLMWC) was finally obtained after collecting the precipitate and drying it over phosphorus pentoxide under vacuum.

The percent yield of acid-free-water-soluble low-molecular-weight chitosan (AFWSLMWC) was calculated as follows:Percent yield of AFWSLMWC = AFWSLMWC (g)/ initial chitosan (g)] × 100%.

3. Results and discussion

3.1. Effect of reaction conditions on the percent yield of AFWSLMWC

3.1.1. Effect of the reaction temperature on the percent yield of AFWSLMWC

To investigate the effect of reaction temperature on percent yield of AFWSLMWC, 200 ml chitosan solution (0.05 g/ml) were treated with ozone (65 ± 5 mg/min) at different temperatures for 60 min, and the corresponding samples were processed in the same way as described above. The percent yields of AFWSLMWC were plotted as a function of the reaction temperature.

As shown in Fig. 2A, percent yield of AFWSLMWC increased up to 50 °C, but slowly decreased at higher temperatures. Usually, an increase in temperature is accompanied by an increase in the reaction rate. Temperature is a measure of the kinetic energy of a system, so higher temperature implies higher average kinetic energy of molecules and more collisions per unit time. Once the temperature goes above 50 °C, ozone saturation in chitosan solution decreases. Thus the reaction rate decreased in spite of the higher average kinetic energy of molecules. Hence, the optimum reaction temperature was 50 °C.

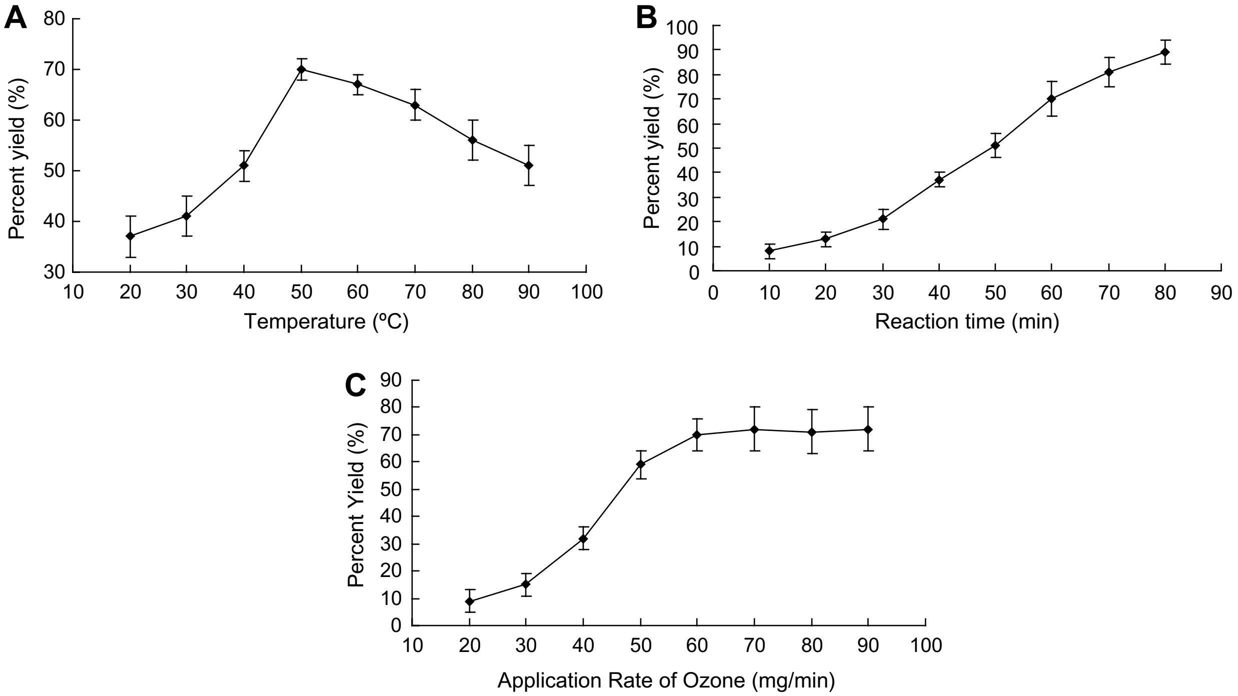

Fig. 2. Effect of reaction conditions on the percent yield of AFWSLMWC. (A) Effect of the reaction temperature, (B) effect of the reaction time and (C) effect of application rates of ozone. All values are the mean ± SD of three independent experiments.

3.1.2. Effect of the reaction time on the percent yield of AFWSLMWC

To investigate the effect of reaction time on percent yield of AFWSLMWC, 200 ml chitosan solution (0.05 g/ml) were treated with ozone (65 ± 5mg/min) at 50 °C for different times, and the corresponding samples were processed in the same way as described above. The percent yields of AFWSLMWC were plotted as a function of the reaction time. The results are illustrated in Fig. 2B.

As shown in Fig. 2B, percent yield of AFWSLMWC obviously increased with increasing reaction time. This is because the hydroxyl radical generated from the active ozone molecule attacked the β-D-(1,4) glucosidic linkages of chitosan and made the linkages break, which decreased the molecular weight of chitosan in a short time. Because the molecular weight became lower and lower by prolonging ozone treatment, the percent yield of AFWSLMWC obviously increased.

3.1.3. Effect of application rate of ozone on the percent yield of AFWSLMWC

To investigate the effect of application rate of ozone on percent yield of AFWSLMWC, 200 ml chitosan solution (0.05 g/ml) were treated with ozone (different application rates of ozone were used) at 50 °C for 60 min, and the corresponding samples were processed in the same way as described above. The percent yields of AFWSLMWC were plotted as a function of application rates of ozone. The results are illustrated in Fig. 2C.

The percent yield of AFWSLMWC was found to increase with increasing ozone flow rate, ranging from 20 to 60 mg/min. This is because the increase in ozone concentration was accompanied by an increase in the reaction rate. The percent yield did not increase when the ozone flow rate exceeded 60 mg/min because the solution was completely saturated with ozone.

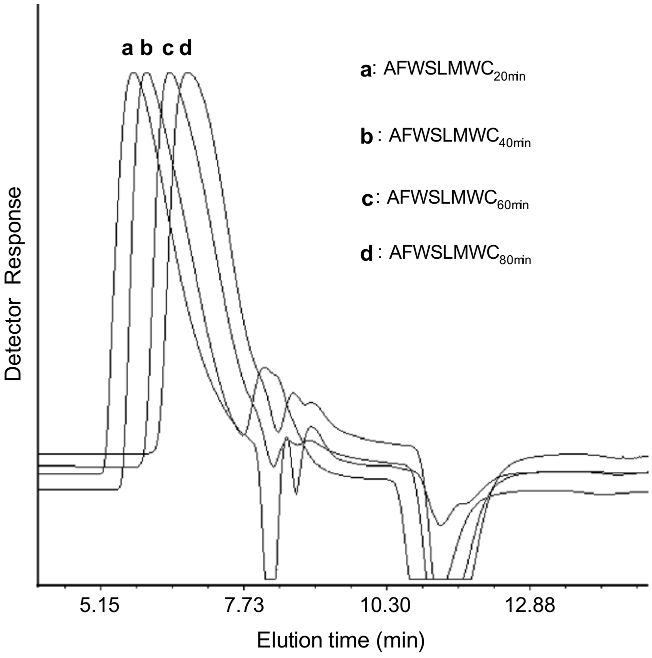

Fig. 3. GPC curves of four kinds of acid-free-water-soluble chitosans.

3.2. Molecular weight distribution of AFWSLMWC

Gel permeation chromatography, often referred to as size exclusion chromatography, is perhaps the most widely-used method for the molecular weight and molecular weight distribution characterisation of polymers. Fig. 3 shows the GPC curves of AFWSLMWC samples obtained after different reaction times. The shift toward higher elution time as a consequence of the degradation could be observed for all AFWSLMWC samples. Longer elution times correspond to a decrease in the molecular weight of AFWSLMWC. Obviously, as was also shown in Table 1, the extent of degradation of AFWSLMWC was increased by prolonging the duration of ozone treatment.

3.3. FT-IR spectra of hydrochloride salts of AFWSLMWC as well as initial chitosan

FT-IR spectroscopy has been shown to be a powerful tool for the study of the physicochemical properties of polysaccharides. The spectra of hydrochloride salts of AFWSLMWC, as well as initial chitosan, are shown in Fig. 4. In the spectrum of the hydrochloride salt of AFWSLMWC, the characteristic peaks of $-NH_3^+$ vibration deformation appeared at 1622.05 and 1516.97 cm^{-1} which were attributed to the presence of amide I (νC=O) and amide II (δN–H), respectively. For AFWSLMWC, the relative intensity of the $-NH_3^+$ related band at 1516.97 cm^{-1} is nearly the same as that of the initial chitosan. As can be seen, the spectral profiles of AFWSLMWC are similar to those of the initial chitosan though some insignificant differences do exist. Thus the conclusion can be drawn that the whole initial chitosan's monomeric structure still existed in the resulting acid-free-water-soluble chitosan with reduced molecular weight. No new band at about 1735 cm^{-1} assigned to the carboxylic group was obviously seen. The results also give us the important information that the reaction was mainly the cleavage of β-glycosidic linkages, and no carboxylic group was formed.

Table 1
M_w, M_w/M_n, and DD of AFWSLMWC as well as initial chitosan

WSLMWC Sample	M_w/kDA	M_w/M_n	DD (%)
Initial chitosan	245.9	3.744	89.8 ± 1.8*
WSLMWC$_{20min}$	13.1	1.728	89.7 ± 1.6
WSLMWC$_{40min}$	8.2	1.875	90.2 ± 1.3
WSLMWC$_{60min}$	6.1	1.670	89.5 ± 1.4
WSLMWC$_{80min}$	4.3	1.674	89.9 ± 1.2

* Mean ± standard derivation. The meaning of subscript is the reaction time for preparation of AFWSLMWC samples.

3.4. The degree of deacetylation of AFWSLMWC

The DD percentages of AFWSLMWC (Table 1) did not apparently change with decreasing molecular weight, suggesting that the protonated amino groups are stable enough to the attack by hydroxyl radicals generated from ozone and that glycosidic bonds might be randomly cleaved.

4. Conclusions

Acid-free-water-soluble low-molecular-weight chitosan can be obtained by ozone treatment. The method is promisingly suitable for scale-up manufacture of acid-free-water-soluble low-molecular-weight chitosan. In batch experiments on a laboratory scale, the optimum reaction temperature was 50 °C and the optimum application rate of ozone was 60 mg/min. The DD of the acid-free-water-soluble low-molecular-weight chitosan did not apparently change with decreasing molecular weight. IR spectra demonstrated that the chemical structures of acid-free-water-

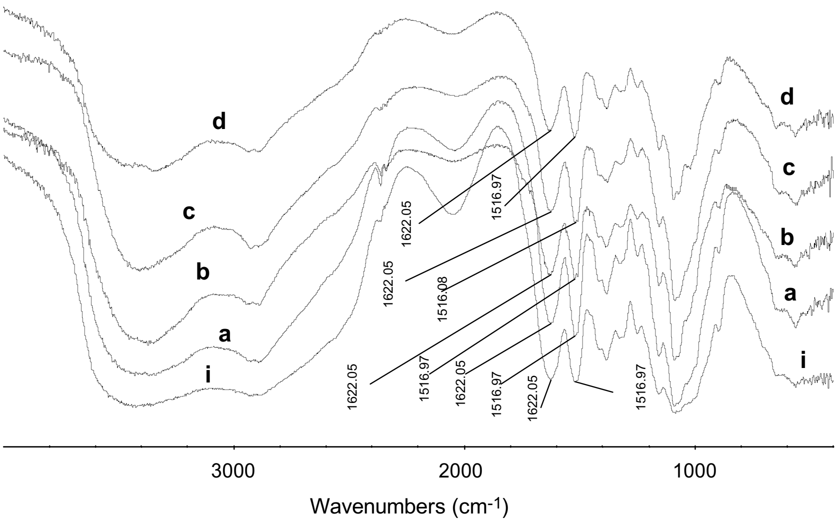

Fig. 4. FT-IR spectra of hydrochloride salts of AFWSLMWC as well as initial chitosan. (i) Initial chitosan, (a) AFWSLMWC$_{20min}$, (b) AFWSLMWC$_{40min}$, (c) AFWSLMWC$_{60min}$ and (d) AFWSLMWC$_{80min}$.

soluble low-molecular-weight chitosan were not modified during the depolymerisation process.

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