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Preparation of low-molecular-weight hyaluronic acid by ozone treatment

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ABSTRACT

Recently, low-molecular-weight hyaluronic acid has been reported to have novel features, such as free radical scavenging activities, antioxidant activities, promotion of excisional wound healing, etc. In the present work, degradation of native hyaluronic acid by ozone treatment was performed for preparation of low-molecular-weight hyaluronic acid. The molecular weight of native hyaluronic acid was reduced from 1535 to 87 kDa for 120 min at 40 °C. The rate of reduction of molecular weight was 94.33%. The FT-IR, ^{13}C NMR, and UV–vis spectra suggested that there was no obvious modification of chemical structure of low-molecular-weight hyaluronic acid. The use of degradation of native hyaluronic acid by ozone treatment can be a useful alternative for production of low-molecular-weight hyaluronic acid.

1. Introduction

Hyaluronic acid, also known as hyaluronan or hyaluronate, is a linear glycosaminoglycan composed of disaccharide repeating units, namely, [→4)-beta-D-GlcpA-(1 → 3)-beta-D-GlcpNAc-(1→]. Traditionally, native hyaluronic acid was extracted from animal tissues like rooster combs, umbilical cord, bovine vitreous humor, etc. However, mainly due to the limited tissue sources and risk of viral infection, the traditional extraction techniques is increasingly being replaced by the microbial fermentation. Compared with the traditional extraction method, the microbial fermentation, more specifically the optimized microbial fermentation has the advantages of high purification efficiency, low production cost, and low risk rate of cross-species viral infection (Izawa, Hanamizu, Sone, & Chiba, 2010; Izawa, Serata, Sone, Omasa, & Ohtake, 2011; Liu, Du, Chen, Wang, & Sun, 2008; Liu, Wang, Sun, Du, & Chen, 2010). Owing to its unique physico-chemical properties and biological activities, the native hyaluronic acid has been used in a wide variety of applications, such as food, biomedicine, biomaterials, and cosmetics. In recent years, it has been reported that low-molecular-weight hyaluronic acids reveal significantly different biological activities (Stern, Asari, & Sugahara, 2006). For example, the hyaluronic acids with an average molecular weight in the range of 45.2–145 kDa were shown to possess pronounced free radical scavenging and antioxidant activities, particularly compared to the native hyaluronic acid of 1050 kDa (Ke, Sun, Qiao, Wang, & Zeng, 2011). It has also been reported that gamma irradiation of the native hyaluronic acid could increase its antioxidant activity as a result of a decrease in molecular weight (Kim et al., 2008). Low-molecular-weight hyaluronic acids are also effective angiogenic factors (Cui et al., 2009) and can promote excisional wound healing through enhanced angiogenesis (Gao et al., 2010). There is evidence to indicate that low-molecular-weight hyaluronic acids inhibit colorectal carcinoma growth by decreasing tumor cell proliferation and stimulating immune response (Alaniz et al., 2009). Furthermore, low-molecular-weight hyaluronic acids could be considered a new adjuvant candidate in the preparation of dendritic cells-based anticancer vaccines with potent immunostimulatory properties (Alaniz et al., 2011).

Thus it is necessary to convert the native hyaluronic acid into the low-molecular-weight hyaluronic acid. At present, many methods that can cause degradation of the native hyaluronic acid have been presented (Stern, Kogan, Jedrzejas, & Šoltés, 2007), including physical method (Gu, Cai, He, Fu, & Li, 2010; Kubo, Nakamura, Takagaki, Yoshida, & Endo, 1993; Kwon, Hwang, Cho, & Moon, 2009; Miyazaki, Yomota, & Okada, 2001), chemical approach (Rychlý et al., 2006; Šoltés et al., 2007; Yamazaki et al., 2003), and enzymatic process (Chen et al., 2009; El-Safory, Fazary, & Lee, 2010; Gao, Cao, Yang, He, & Liu, 2006; Lenormand, Amar-Bacoup, & Vincent, 2011; Liu et al., 2009).

Without doubt, the degradation methods mentioned above have laid foundations for further research on preparation of low-molecular-weight hyaluronic acid.

The aim of the present work is to present a new method for preparation of low-molecular-weight hyaluronic acid by ozone treatment and to characterize its chemical structure.

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2. Materials and methods

2.1. Materials

Food-grade native hyaluronic acid (1535 kDa) was purchased from Dali Hyaluronic acid Co., Ltd. of Liuzhou Chemical Group (Guangxi, China). Oxygen purity is over 99%. All other chemicals were of reagent grade.

2.2. Characterization of low-molecular-weight hyaluronic acid

Molecular weight was calculated on the basis of the relationship between the limiting viscosity number and light-scattering molecular weight (Laurent, Ryan, & Pietruszkiewicz, 1960):

$$[\eta] = 0.036M^{0.78}$$

where $[\eta]$ and M denote the limiting viscosity number and the light-scattering molecular weight, respectively.

Polymolecularity (Mw/Mn) of hyaluronic acid were measured by gel permeation chromatography (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 $NaNO_3$. The flow rate was maintained at 1.0 ml/min. The column temperature was maintained at 30 °C. The standards used to calibrate the column were Tosoh pullulan (Tosoh Corporation, Tokyo, Japan). All data provided by the GPC system were collected and analyzed using the Jiangshen Workstation (Dalian, China) software package.

FT-IR spectra were recorded in the wavelength region between 4000 and 400 cm^{-1} on a Nicolet 5DXB FT-IR spectrophotometer. 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.

^{13}C NMR spectra were recorded on BRUKER AVANCE-500NMR SPECTROMETER. The samples were dissolved in D_2O.

UV–vis absorption spectra were carried out using a UV–vis recording spectrophotometer (UV-2501PC) in the range of 200–400 nm. Distilled water was used as a reference.

2.3. Method for preparation of low-molecular-weight hyaluronic acid

Solutions of native hyaluronic acid were prepared by dissolving the hyaluronic acid in 0.15 mol/L sodium chloride solution to a final hyaluronic acid concentration of 1% (w/v). 500 ml solution of native hyaluronic acid was treated with ozone in a reactor (D 8 cm × H 15 cm). Because of its relatively short half-life, ozone was generated on-site by an ozone generator and then was bubbled into the bottom of the reactor through a thin bubble diffuser. In order to provide sufficient contact between the native hyaluronic acid solution and the ozone gas bubble, a stirrer with 1100 rpm mixing speed was used. The reaction temperature was kept at 40 °C. The concentration of ozone was measured by passing the ozone/oxygen gas mixture through a potassium iodide trap and titrating with standardized sodium thiosulfate. The application rate of ozone was 46 ± 5 mg/min. After the ozone treatment process, the treated hyaluronic acid solution was concentrated with a rotary evaporator under reduced pressure. The concentrate was precipitated by adding absolute alcohol. To wash the formed precipitates thoroughly, the precipitates was suspended in 200 ml absolute alcohol at 25 °C for 2 h with constant agitating rate and then collected by filtration. The low-molecular-weight hyaluronic acid was finally obtained after collecting the precipitate and drying it over phosphorus pentoxide under vacuum.

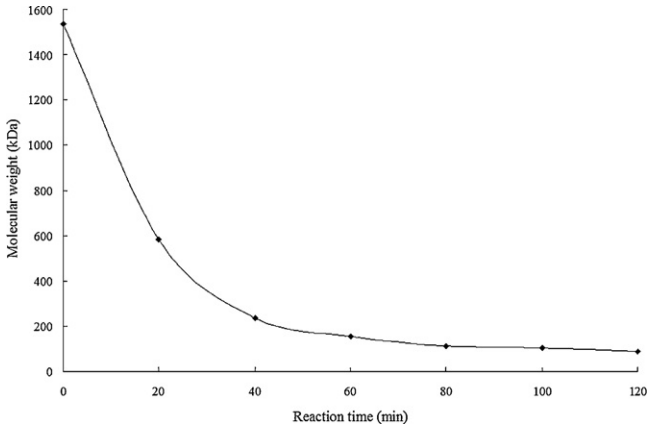

Fig. 1. Effect of ozone treatment on molecular weight of native hyaluronic acid.

3. Results and discussion

3.1. Effect of ozone treatment on molecular weight of native hyaluronic acid

In Fig. 1, as can be seen, the curve shows the changes in molecular weight of native hyaluronic acid treated with ozone. The low-molecular-weight hyaluronic acids of 583, 237, 155, 112, 103, and 87 kDa were prepared after reaction times of 20, 40, 60, 80, 100, and 120 min, respectively. The molecular weight of native

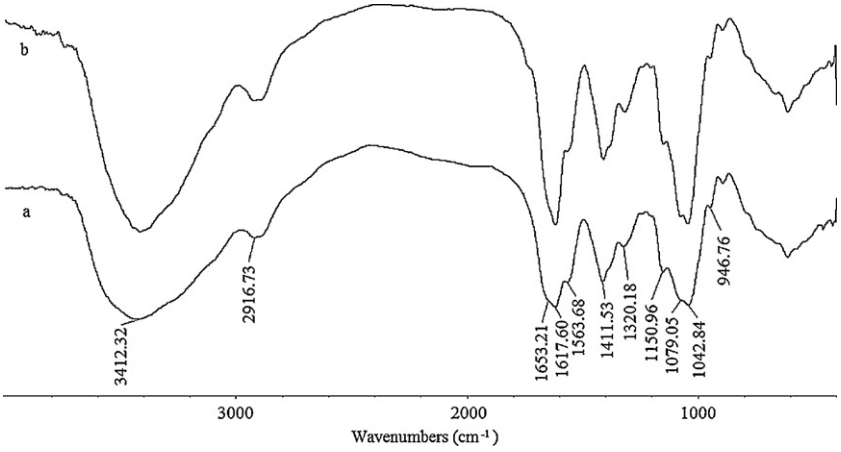

Fig. 2. FT-IR spectra. (a) Native hyaluronic acid of 1535 kDa; and (b) low-molecular-weight hyaluronic acid of 87 kDa.

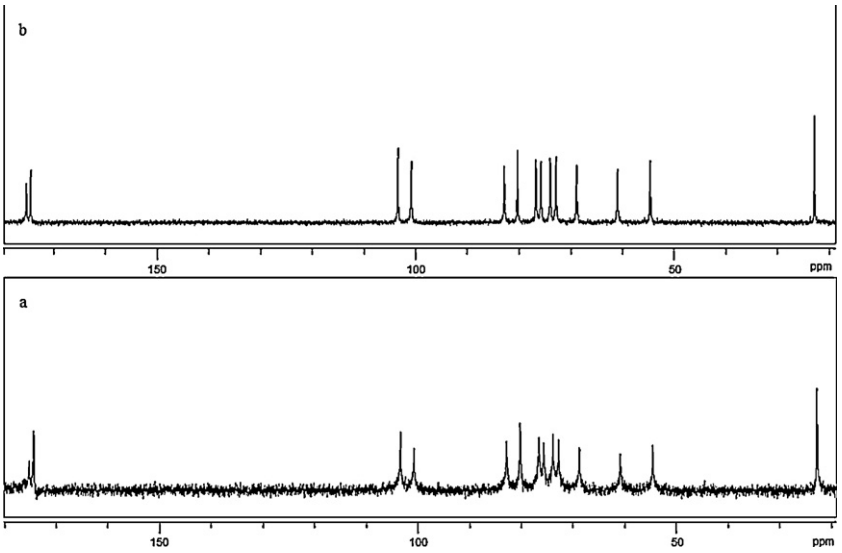

Fig. 3. ^{13}C NMR spectra. (a) Native hyaluronic acid of 1535 kDa; and (b) low-molecular-weight hyaluronic acid of 87 kDa.

hyaluronic acid was reduced from 1535 to 87 kDa for 120 min at 40 °C. The rate of reduction of molecular weight was 94.33%. The degradation of native hyaluronic acid proceeded rapidly during the first 40 min and then slowly leveled off with prolonged ozone treatment (40–120 min). Consequently, the molecular weight values converged to about 87 kDa. It seems that the hyaluronic acid with high molecular weight was more preferentially depolymerized than that with low molecular weight. When ozone was continuously bubbled into the native hyaluronic acid solution, there was much opportunity for the ozone attacking glucosidic linkages of the hyaluronic acid and making the linkages break. Consequently, the molecular weight of hyaluronic acid decreased. The conclusion can be drawn that the native hyaluronic acid treated with ozone can be converted into the low-molecular-weight hyaluronic acid.

After reaction times of 20, 40, 60, 80, 100, and 120 min, the polymolecularity of low-molecular-weight hyaluronic acids is 3.46, 4.73, 3.24, 2.49, 1.89, and 1.66, respectively. The polymolecularity of native hyaluronic acid was increased from 1.94 to 4.73 for 40 min and then decreased from 4.73 to 1.66 (40–120 min). The changes in polymolecularity of hyaluronic acid may be due to the heterogeneous reaction between gas-phase ozone and hyaluronic acid in solution.

It was also found that many factors such as reaction temperature, ionic strength, ozone concentration, agitation speed, pH value, etc. all have certain impacts on the degradation reaction of native hyaluronic acid by ozone treatment. To a large extent, the degradation of hyaluronic acid depends on the factors mentioned above. The kinetics of degradation of native hyaluronic acid by ozone treatment is now being intensively investigated.

3.2. Characterization of low-molecular-weight hyaluronic acid

As shown in Fig. 2, the FT-IR spectrum of the low-molecular-weight hyaluronic acid is similar to that of the native hyaluronic acid. The strong band at about 3412.32 cm^{-1} is rather broad and can be assigned to hydrogen-bonded O—H and N—H stretching vibrations. A group of overlapping bands of moderate intensity is observed around 2916.73 cm^{-1} which are due to the C—H stretching vibrations. The bands at 1617.60 and 1411.89 cm^{-1} can be attributed to the asymmetric (C═O) and symmetric (C—O) stretching modes of the planar carboxyl groups in the hyaluronate (Gilli, Kacuráková, Mathlouthi, Navarini, & Paoletti, 1994). These authors explained the form of the carboxyl group by adding "sodium hyaluronate" to their definition, and in the continuing text, the IR bands of the protonated COOH are described: "After protonation these peaks are shifted to 1735 cm^{-1} and 1255 cm^{-1}, respectively." The absorption bands at about 1653.21, 1563.68, and 1320.18 cm^{-1} are characteristic of the amide I, II, and III band, respectively. The

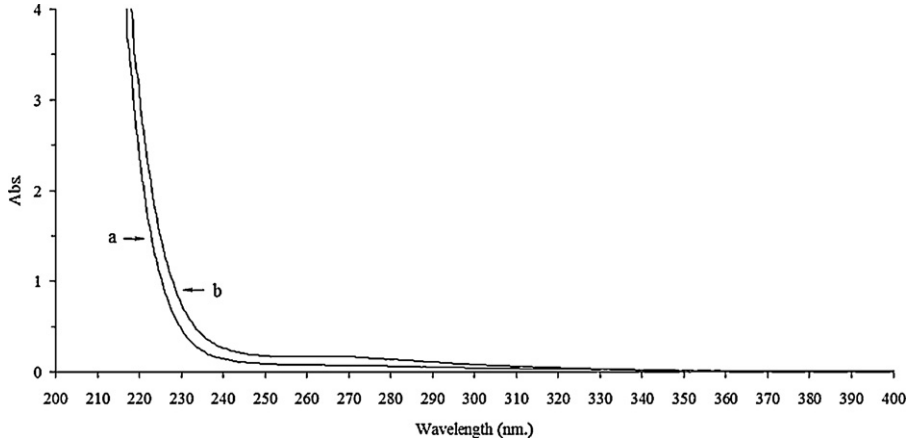

Fig. 4. UV–vis spectra. (a) Native hyaluronic acid of 1535 kDa; and (b) low-molecular-weight hyaluronic acid of 87 kDa.

three signals centered at 1150.96, 1079.05, and 1042.84 cm^{-1} are assigned to C—O—C (O-bridge), C—O (exocyclic), and C—OH group, respectively. The band at 946.76 cm^{-1} can be assigned to asymmetrical out-of-phase ring vibration. The band resulting from C—C had disappeared in the FT-IR spectra mainly because it overlap with the band of C—O (exocyclic) vibration. As can be observed, the overall spectral pattern did not significantly change before and after degradation of native hyaluronic acid by ozone treatment. The assignments of the peaks mentioned above were based on the data reported in the literature (Alkrad, Mrestani, Stroehl, Wartewig, & Neubert, 2003; Bezáková et al., 2008; Choi, Kim, Kim, Kweon, & Lee, 2010; Dřímalová, Velebný, Sasinková, Hromádková, & Ebringerová, 2005; Luan, Wu, Zhang, & Wang, 2012).

To further confirm its structural change, the low-molecular-weight hyaluronic acid was analyzed by ^{13}C NMR and UV–vis spectroscopy. The ^{13}C NMR spectra are shown in Fig. 3. It can be seen that the low-molecular-weight hyaluronic acid (b) has the almost same signals as the native hyaluronic acid (a). The UV–vis spectra are shown in Fig. 4. As can be observed, the UV–vis spectra of the low-molecular-weight hyaluronic acid (b) and the native hyaluronic acid (a) almost overlap. There are no significant differences between the UV–vis spectra before and after degradation of native hyaluronic acid by ozone treatment.

The FT-IR, ^{13}C NMR, and UV–vis spectra suggested that there was no significant difference between the chemical structure of the low-molecular-weight hyaluronic acid and native hyaluronic acid. Thus the conclusion can be drawn that when the reaction temperature was kept at 40 °C, the whole monomeric structure was well preserved in the low-molecular-weight hyaluronic acid and the degradation reaction was mainly the cleavage of glycosidic linkages.

4. Conclusions

The preparation of low-molecular-weight hyaluronic acid can be performed through degradation of native hyaluronic acid by ozone treatment. The FT-IR, ^{13}C NMR, and UV–vis spectra demonstrated that the chemical structure of low-molecular-weight hyaluronic acid was not modified during the degradation process. The use of degradation of native hyaluronic acid by ozone treatment can be a useful alternative for production of low-molecular-weight hyaluronic acid. The method is promisingly suitable for the scale-up manufacture of low-molecular-weight hyaluronic acid.

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