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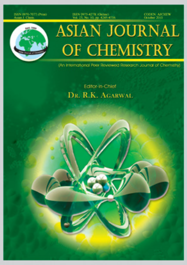

Degradation of Hyaluronic Acid by Multiple Ultrasonic

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Ultrasonic degradation of hyaluronic acid salt solutions was investigated by a ultrasonic instrument, which is designed to make use of multiple ultrasonic oscillator simultaneously. Single source, two sources and three sources were irradiated at high frequency (1.7 MHz) and low frequency (800 kHz). The degraded samples were analyzed by gel permeation chromatography for the time evolution of the molecular weight distributions and the contents of $OH^•$ radicals were obtained as functions of sonication time. A continuous distribution kinetics model based on midpoint chain scission was developed and the limiting molecular weight and degradation rate were determined. The experimental data showed that more $OH^•$ radicals were produced in the unit time when it was irradiated with two or three sources compared with the single source, which means the degradation rate of the hyaluronic acid increased. The limiting molecular weight was decreased by multiple ultrasonic treatment. This implies that the ultrasonic degradation of hyaluronic acid is faster and more effective.

Key Words: Multiple ultrasonic, Free radical reaction, Molecular weight, Hyaluronic Acid.

INTRODUCTION

Hyaluronic acid is a linear anionic polysaccharide consisting of repeating disaccharide units of D-glucuronic and 2-acetamido-2-deoxy-D-glucose, which are bound together by β-(1→3)-type glycosidic bond. Degradation of hyaluronic acid occurs due to heat, light, chemical reagents and ultrasonic radiation[1]. The ultrasonic degradation of hyaluronic acid in solutions, unlike other chemical and thermal degradation processes, is a nonrandom process in which the polymer molecule undergoes scission preferentially at the midpoint of the chain. Degradation of aqueous solutions provokes the formation and collapse of cavitation bubbles[2]. During the collapse high temperatures and pressures are obtained, leading to the formation of chemical species such as $H^•$, $OH^•$, $O^•$ and H_2O_2. Those high-energy phenomena cause degradation of organic compounds in aqueous solutions[3]. Basically, the degradation proceeds by two reaction mechanisms such as direct pyrolysis in and around the collapsing bubbles and oxidation by $OH^•$ radicals. There exists a limiting molecular weight (x_l) for a polymer below, in which the degradation by ultrasound does not occur.

It is not easy to detect directly pyrolysis intuitive. But the free radical reaction, mainly the $OH^•$ radical reaction in water, can now be measured by the method based on salicylic acid and can be resolved into 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2, 5-DHBA). Detecting is done by high performance liquid chromatography with electrochemical detector system (HPLC-ECD)[4].

Traditional method of ultrasonic degradation applies only a single wave source with one frequency. But the use of ultrasonic radiation is restricted because the limiting molecular weight of a polymer is much higher and the process of ultrasonic degradation is longer than enzymatic. Therefore, multiple ultrasonic degradation with large-scale reverberation ultrasonic sources is attempted. We designed an instrument that can separately use numbers of ultrasonic sources simultaneously (Fig. 1). It contains a ultrasonic wave generator and a flume with holes on the four walls, which is fit for circular ultrasonic oscillator and one ultrasonic plate adhering to the bottom. One, two, three or even more ultrasonic oscillators can emits ultrasound at the same time at different frequencies. The range of ultrasonic oscillator frequency is 800 kHz to 1.7 MHz.

In this study, to demonstrate the advantages of equipment in the degradation, hyaluronic acid is degraded by single source, two sources and three sources. The $OH^•$ radical reaction was obtained by HPLC-ECD. The kinetics of degradation was studied, which was compared with the number-average molecular weight (M_n) detected by the gel permeation chromatography.

Theoretical model: A theoretical model based on continuous distribution kinetics satisfactorily describes the ultrasound degradation of the polymer involving a free-radical mechanism based on Rice-Herzfeld kinetics[5]. The proposed radical mechanism for the degradation of the polymer can be given as follows:

$$P(x) \underset{k_t}{\overset{k_i}{\rightleftharpoons}} R^*(x') + R^*(x-x') \quad \text{(I)}$$

$$P(x) \underset{k_H}{\overset{k_h}{\rightleftharpoons}} R^*(x') \quad \text{(II)}$$

$$R^*(x) \xrightarrow{k_s(x)} P(x') + R^*(x-x') \quad \text{(III)}$$

Reaction (I) represents the initiation and termination steps, in which polymer P(x) of molecular weight x breaks into two radicals of molecular weights x' and (x-x'). The initiation and termination rate coefficients are denoted as k_i and k_t, respectively. The reversible intermolecular hydrogen abstraction is represented by reaction (II) caused by free radical reaction. As polymer P(x) and radical R*(x) differ only by a hydrogen atom, their molecular weights are assumed to be identical. Reaction (III) depicts the depropagation reaction, with the depropagation reaction rate constant $k_s(x)$. Reaction (I) is necessary for the complete mechanism, but its effect on the overall reaction is negligible and can be neglected according to the long-chain reaction approximation[6].

Fig. 1. Instrument of multiple ultrasonic

Continuous distribution kinetics considers molecular weight x as a continuous variable. The time-dependent molecular weight distributions of the polymer and polymer radical are given by p(x,t) and r(x,t), respectively, where p(x,t)dx denotes the molar concentration of the polymer in the interval [x, x+dx]. The population balance equations for polymer P(x), polymer radical R*(x) can be written as follows:

$$\frac{\partial p(x,t)}{\partial t} = -k_h p(x,t) + k_H r(x,t) - k_s(x) r(x,t) + \int_x^{\infty} k_s(x') \Omega(x,x') r(x',t) dx \quad (1)$$

$$\frac{\partial r(x,t)}{\partial t} = k_h p(x,t) - k_H r(x,t) - k_s(x) r(x,t) + \int_x^{\infty} k_s(x') \Omega(x,x') r(x',t) dx \quad (2)$$

where $\Omega(x,x')$ represents the symmetric stoichiometric kernel representing a reaction in which a molecule of molecular weight x breaks into two progenies of molecular weights x' and x-x'. The stoichiometric kernel for scission occurring at mid-chain, as in ultrasonic degradation, is given by the dirac delta function: $\Omega(x,x') = \delta(x - x'/2)$. The rate coefficients for hydrogen abstraction, k_h and k_H, are assumed to be independent of the molecular weight. $k_s(x)$ is assumed to be a linear function of x: $k_s(x) = k_s(x-x')$. This relationship ensures that the rate coefficient becomes zero when the molecular weight reaches xl and no further degradation takes place.

Applying the moment operation $f^n(t) = \int_0^{\infty} x^n f(x,t) dx$, eqs. can be obtained by the fllowing:

$$\frac{dp^{(n)}(t)}{dt} = -k_h p^{(n)}(t) + k_H r^{(n)}(t) + \frac{k_s}{2^n}\left[r^{(n+1)}(t) - x_1 r^{(n)}(t)\right] \quad (3)$$

$$\frac{dr^{(n)}(t)}{dt} = k_h p^{(n)}(t) - k_H r^{(n)}(t) + \frac{k_s}{2^n}\left[r^{(n+1)}(t) - x_1 r^{(n)}(t)\right] - k_s\left[r^{(n+1)}(t) - x_1 r^{(n)}(t)\right] \quad (4)$$

The zero and first moments, $p^{(0)}(t)$ and $p^{(1)}(t)$, represent the molar and mass concentrations, respectively. The initial conditions for the moments are $p^{(0)}(t = 0) = p^{(0)}$, $r^{(0)}(t = 0) = 0$ If we apply the quasi-steady-state assumption, the rate of change of polymer radicals can be neglected because their concentration is extremely small. Thus, $\frac{dr^{(0)}(t)}{dt} = 0$ and $r^{(0)} = \frac{k_h}{k_h}$. So equation is solved to obtain:

$$\frac{dp^{(0)}(t)}{dt} = k_s\left[r^{(1)}(t) - x_1 p^{(0)}(t)\right] \quad (5)$$

Because $\frac{dr^{(1)}(t)}{dt} = 0$ and $r^{(2)}(t)$ is assumed to be small and the second term is neglected in the expression for $r^{(1)}(t)$, there is an equation:

$$r^{(1)}(t) = \frac{k_h}{\left[1 - \frac{x_1 k_s}{2k_h}\right] k_h} p^{(1)}(t) \quad (6)$$

As $t \to \infty$, $M_n \to x_l$, and this implies $\frac{x_1 k_s}{2k_H} << 1$, the simplified expression for $r^{(1)}(t)$ is:

$$r^{(1)}(t) = \frac{k_h}{k_H} p^{(1)}(t) \quad (7)$$

Using $r^{(1)}(t)$, eqn. (5) can be solved with the initial condition $p^{(0)}(t = 0) = p_0^{(0)}$ to give:

$$p^{(0)}(t) = \frac{p^{(1)}(t)}{x_1} + \left[-\frac{p^{(1)}(t)}{x_1} + p_0^{(0)}\right] \times \exp\left(-\frac{k_s k_h}{k_H} x_1 t\right) \quad (8)$$

Eqn. (8) can be rewritten as:

$$\ln \frac{\left(\frac{1}{x_1} - \frac{1}{Mn_0}\right)}{\left(\frac{1}{x_1} - \frac{1}{Mn}\right)} = k_1 x_1 t \quad (9)$$

TABLE-1
PEAK AREA AND HEIGHT OF 2,3-DHBA FOR FOUR METHODS

Reaction Time (h)	One source (800 kHz)		One source (1.7 MHz)		Two sources		Three sources	
	Height	Area	Height	Area	Height	Area	Height	Area
12	54656±212	2313494±9865	62031±330	2435422±7823	72134±298	3034454±8212	74345±192	3283797±9032
24	72489±332	3372662±9084	72123±213	3442355±8931	89213±291	3921312±7821	93834±231	4132343±7261

where,

$$Mn = \frac{p_0^{(1)}}{p_0} \tag{10}$$

M_n and M_{n_0} represent the number-average molecular weights at times t and t = 0, respectively. For the hyaluronic acid, x_l is usually much less than Mn_0, so the equation can be further simplified into:

$$\ln \frac{M_n}{M_n - x_l} = \frac{x_l k_s k_h t}{k_h} \tag{11}$$

It can be obtained M_n-t curve by experimental, then x_l can be calculated. Compared x_l calculated with final M_n from experiment and analysis of M_n-t curves, the efficiency of the ultrasonic is determined.

EXPERIMENTAL

Hyaluronic acid with a viscosity-average molecular weight of 9,900,000 Da was purchased from Seikagaku (Tokyo Japan). The number average molecular weight, as determined by gel permeation chromatography analysis, were 9,840,000 Da. Acetonitrile (HPLC grade, SCRC), methanol (HPLC grade, SCRC), phosphoric acid (>85 %, SCRC), ethanol (HPLC grade, SCRC), sodium acetate trihydrate (AR, SCRC), citric acid (AR, SCRC), disodium EDTA (AR, SCRC) and galkyl sulfonate (AR, SCRC) were used without further purification.

Degradation experiments: All ultrasonic degradation experiments were carried out in a 500 mL glass beaker. The 5 g hyaluronic acid dissolved in 500 mL 0.2 M NaCl solution was maintained at 5 ± 0.1 ºC with a thermostated water bath. Various compositions of the frequency and intensity were used to observe the effect of quantity of sources on the degradation. The samples were respectively irradiated by three methods. With one ultrasound source, the frequency was set at 1.7 MHz and 800 kHz respectively. With two sources, the frequency was set at 1.7 MHz vertically and 800 kHz horizontally together. For the three sources, two were set in adjacent surfaces at a frequency of 800 kHz and another was set at a frequency of 1.7 MHz. Ultrasonic disposed of hyaluronic acid for 24 h at maximum output power of the equipment: 23 W/cm_2.

High performance liquid chromatography-electrochemical detector analysis: The 0.2 M NaCl solution without hyaluronic acid was treated with the three methods, reacting with 500 mg salicylic acid in a 500 mL glass beaker. HPLC-ECD analysis was then performed using a NANOSPACE SI-2 HPLC system equipped with an eight-channel coulometric array detector (5600 A, ESA) and a SHISEIDO MG_2-C_{18} column (150 nm × 4.6 mm). The optimized electric potential of each component was 240 mV for 2,3-dihydroxy benzoic acid, 150 mV for 2,5-dihydroxy benzoic acid and 650 mV for salicylic acid[7].

Gel permeation chromatography: Molecular weight was obtained by GPC with following system: a separation module (Agilent-1100, Agilent Co., US), a refractive index detector (G1362A, Agilent Co.), System Software (Agilent-1100, Agilent Co.,) and TSK-GEL G3000 PW_{XL} and G4000 PW_{XL} (TOSOH, Tokyo, Japan). The mobile phase was 0.2 M sodium nitrate at flow rate of 1 mL/min and the analyses were performed at 40 ºC. The injection volume was 100 µL[8].

RESULTS AND DISCUSSION

Shinobu and Kimihiko[9] have investigated that with the power and frequency increasing, the degradation rate accelerated and x_l reduced. However, low-frequency ultrasound is more penetrating. So we choose the maximum frequency and low-frequency to test the joint use.

$OH^•$ radical reaction: Eqn. 11 gives the relationship between M_n and the sonication time in terms of x_l and k_h. This equation shows the effect of the content of free radicals and limiting molecular weight on the ultrasonic degradation of hyaluronic acid. In a certain period, the more free radicals produced, the lower the molecular weight of the hyaluronic acid.

To study the effects of different ultrasonic combinations by detecting the hydroxy radical with time, hyaluronic acid was degraded. The experimental data were plotted on the peak area and height of 2,3-dihydroxy benzoic acid (Table-1). In all cases modeled in this study, the regression coefficient was higher than 0.95. Obviously, in the 12 and 24 h, source with 1.7 MHz attain more 2,3-dihydroxy benzoic acid than source with 800 kHz and even more OH· is produced by two and three sources. Based on the above observation, two inference is drawn: high-frequency ultrasound is more effective; the combined with high-frequency and low frequency can greatly increase rate.

The cavitation occurs at a range of frequency. Single source only leads to a fixed frequency of cavitation, while multi-frequency waves in the superposition of some point will expand the frequency coverage and then greatly increase the probability of cavitation. As low-frequency ultrasound can cause surface resonance[8] and high-frequency ultrasound can come into being through large amounts of energy[10], multiple ultrasonic puts together the advantages of both.

Change of molecular weight: Fig. 2 shows the variation of M_n with time. The values of final Mn obtained from Fig. 2 for four methods are 127320, 98400, 13430 and 9650 Da. Average x_l from eqn. 11 were 23323, 25419, 14654 and 129fc21 Da. When two or three sources were used, final Mn was closer to the average x_l than one source. Although there exited some fragments of polymer (Fig. 3), molecular weight distribution was narrow.

An important factor of ultrasonic degradation is the surface area between the ultrasound and polymers[11]. Multiple

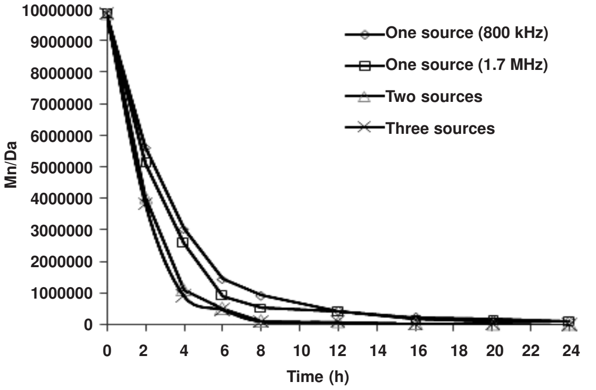

Fig. 2. Number-average molecular weight as a function of sonication time.

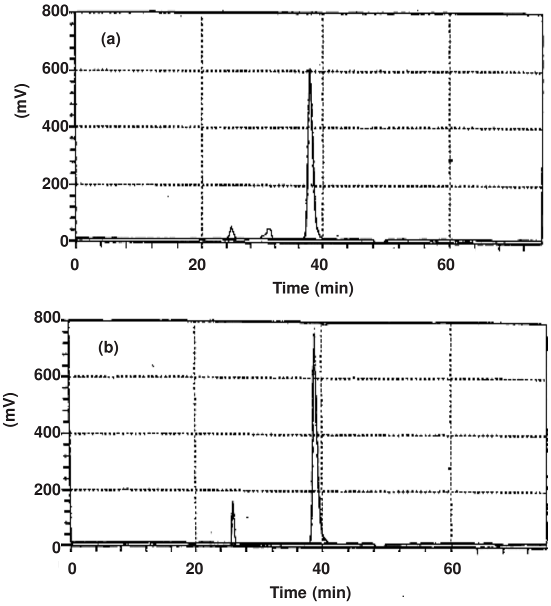

Fig. 3. RI absorption of gel permeation chromatography after ultrasonic irradiation for 24 h. a: degradation by two sources; b: degradation by three sources.

ultrasonic enables more air to penetrate into the sample from the sample surface, leading to increase in cavitation nuclei. More mechanical disturbance is added to the reaction, resulting in the reduction of the cavitation threshold.

Conclusion

Continuous distribution kinetics has been applied to model the experimental data and determine the relationship between content of free radicals and degradation rate. The rate of degradation of hyaluronic acid increased with two or three large-scale reverberation sources and the limiting molecular weight decreased. This indicates that polymers can be more effectively degraded by multiple ultrasonic instrument.

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