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Degradation of hyaluronic acid powder by electron beam irradiation, gamma ray irradiation, microwave irradiation and thermal treatment: A comparative study

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ABSTRACT

In this study, high molecular weight hyaluronic acid in powder form (HMW-HA, with average molecular weight of 1042 kDa) was degraded to low molecular (LMW-HA, 200–230 kDa) by several methods, and the changes in molecular structure and antioxidative activities brought about by each degradation method were compared. The degradation methods used were electron beam irradiation (EB), gamma ray irradiation (GM), microwave irradiation (MW), and thermal treatment (TH). The FT-IR spectra showed no substantial changes of the spectral pattern between HMW and LMW-HAs. However, the UV absorbance of LMW-HA by MW was considerably greater at 265 nm indicating the formation of more double bonds. The antioxidative activities of all LMW-HA samples were found to have risen, but the MW-treated LMW-HA showed the most significant increase due to a newly formed double bond. EB- and GM-treated LMW-HA showed the lowest polydispersity and little change in UV spectra from those of HMW-HA.

1. Introduction

Hyaluronic acid (Hyaluronan, HA) is a large, nonsulfated glycosaminoglycan that has important structural and biological roles within the extracellular matrices surrounding animal cells. It is composed of repeating disaccharide subunits of β1-4-D-glucuronic acid and β1-3*N*-acetyl-D-glucosamine. HA plays an important role in many biological processes and finds many applications in the fields of medicine, pharmaceuticals, and cosmetics (Fraser, Laurent, & Laurent, 1997; Goa & Benfield, 1994; Lapčík, Lapčík, De Smedt, Demeester, & Chrabreček, 1998). This interest is related to its unique physico-chemical properties, such as high intrinsic viscosity, chain stiffness, and water-holding capacity. Recently, low molecular weight HA (LMW-HA) has been reported to have new biological activities not associated with the parent molecule. For example, LMW-HA has been shown to promote angiogenesis (West, Hampson, Arnold, & Kumar, 1985), to induce expression of inflammatory mediators in alveolar macrophages (Horton, Shapiro, Bao, Lowenstein, & Noble, 1999; McKee et al., 1996; Noble, McKee, Cowman, & Shin, 1996), to inhibit tumor growth *in vivo* (Zeng, Toole, Kinney, Kuo, & Stamenkovic, 1998), to activate the NF-κB pathway (Fitzgerald, Bowie, Skeffington, & O'Neill, 2000), to produce transforming growth factor-β in eosinophils (Ohkawara et al., 2000), to stimulate the expression of the cell adhesion molecules ICAM-1 and VCAM-1 in mouse kidney epithelial cells for proinflammatory effects (Oertli, Beck-Schimmer, Fan, & Wuthrich, 1998), and to protect acute hepatotoxicity by suppression of interferon-γ and increase of α-class glutathione-*S*-transferase (GSTs) expression in mice (Kim et al., 2008a).

Various methods for the production of LMW-HA have been described. Ultrasonic degradation is a well established procedure (Mason & Lorimer, 2002), which has been applied on HA by several authors (Gura, Hűckel, & Műller, 1998; Kubo, Nakamura, Takagaki, Yoshida, & Endo, 1993; Orviský, Šoltés, Chabreček, Novák, & Stančiková, 1993). Recently, HA with a broad range of molecular masses was prepared by ultrasonication in neutral aqueous solutions containing various cations (Miyazaki, Yomota, & Okada, 2001) as well as by oxidative degradation using an ascorbic acid/H_2O_2 system (Hokputsa, Jumel, Alexander, & Harding, 2003). HA has also been degraded by exposure to high temperature in an autoclave (Bothner, Waaler, & Wik, 1998), and by acid hydrolysis (Tokita & Okamoto, 1995). Bezáková et al. (2008) studied temperature-controlled microwave-assisted degradation of HA. Ionizing radiation was also used to degrade polymers including HA using the energy generated from different sources such as a cobalt-60 and an electron accelerator (Hayes, Murano, Murano, Olson, & Sapp, 1995; Kim et al., 2008b).

In all of the previous studies, high molecular weight of HA (HMW-HA) was dissolved in water and the solution was used for

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degradation (Bezáková et al., 2008; Kim et al., 2008b; Miyazaki et al., 2001). However, there are many disadvantages when the solution form of HA is used for degradation. The major problem is that the additional processes are necessary to restore the solution to powder form because LMW-HA is sold in powder form on the market. Thus, for industrial applications development of a method for degradation of HMW-HA without having to dissolve it in water was needed. However, the essential requirement is to preserve the fundamental structure of HA during degradation, and to avoid or minimize the formation of new functional groups and/or contamination by side reaction products, which might affect the biological response of the degraded polymer.

Therefore, the purpose of this study was to degrade HMW-HA (1042 kDa) in the powder form to LMW-HA powder (200–230 kDa). Several methods including electron beam irradiation (EB), gamma ray irradiation (GM), microwave irradiation (MW), and thermal treatment (TH) were investigated to achieve this, and the changes brought about in structure and antioxidant activity by each degradation method were compared.

2. Materials and methods

2.1. Materials

Hyaluronic acid powder, extracted from *Streptococcus zooepidecus*, of purity higher than 95% (w/w), was purchased from KOLON Life Science Inc. (Youngin, Republic of Korea). Its weight average molecular weight determined by gel permeation chromatography was 1042 kDa. All chemicals used were of analytical grade.

2.2. Degradation of hyaluronic acid

All degradation experiments were performed in the powder form and degraded samples were stored in a refrigerator at −20 °C for the experiments that followed.

2.2.1. Electron beam irradiation

ELV4-electron accelerator (energy 10 MeV, beam power 570 kW) was used for the electron beam irradiation of the HMW-HA powder. The beam current employed was 1 mA. Irradiation was carried out in air with the distance from the beam source to the sample being 50 cm. HMW-HA powder was irradiated to 10 mm of thickness due to its low penetration. The absorbed dose of 50 kGy in the electron beam irradiation was used to produce LMW-HA powder with specified molecular weight. Dosimetry was carried out using cellulose triacetate film.

2.2.2. Gamma ray irradiation

HA powder contained in tightly capped tubes was irradiated by a cobalt-60 irradiator (point source, AECL, IR-79, Nordion, Canada) at an absorbed dose of 50 kGy. The source strength was approximately 11.1 PBq with a dose rate of approximately 10 kGy/h at the sample location. Irradiation was carried out at 22 ± 0.5 °C. Dosimetry was performed using alanine dosimeters (Bruker Instruments, Rheinstetten, Germany).

2.2.3. Microwave irradiation

The microwave irradiation was carried out in the microwave reactor Initiator EXP (Biotage, Sweden). Microwave frequency was 2.45 GHz and maximum microwave power was 400 W. The time taken for the preparation of LMW-HA with the targeted molecular weight was about 13 h.

2.2.4. Thermal treatment

The thermal treatment was processed in a programmed dry oven (VOS-450VD, ELISA, Japan) for 52 h at 90 °C.

2.3. Gel permeation chromatography (GPC)

For GPC the following system was used: a separation module (Waters 2690, Waters Co., Milford, MA), a refractive index detector (RI, Waters 2410, Waters Co.), Empower software (System Software, Empower option GPC, Waters Co.), and PL aquagel-OH-60, -40, and -30 columns (300 × 7.5 mm, 8 μm, Polymer laboratories Ltd., UK). The mobile phase was 0.1 M sodium nitrate at flow rate of 1 mL/min, and the analyses were performed at 40 °C. The injection volume was 200 μL, and calibration was carried out using pullulan standard (Showa Denko K.K., Tokyo, Japan).

2.4. Hunter color measurement

The HA powders were transferred into a small Petri dish and measured with a Color Difference Meter (Spectrophotometer CM-3500d, Minolta Co. Ltd., Osaka, Japan) by the reflectance method. The instrument was calibrated with standard black and white tiles before analysis.

2.5. FT-IR spectroscopy

The Fourier-transform infrared (FT-IR) spectra were acquired using a Bruker Spectrometer VERTEX 70 (Bruker Optik, Ettlingen, Germany) in the wavelength region between 2000 and 800 cm^{-1}. Samples were prepared as thin films of the HA mixed with KBr at a polymer/KBr ratio (w/w) of 1–100. Obtained spectra were the result of 24 scans at a spectrophotometer resolution of 8 cm^{-1}.

2.6. UV spectrum

HA samples were diluted in distilled water. UV–visible spectroscopy of HA solution was carried out at 25 °C using a spectrophotometer (UV-1601PC, Shimadzu Co., Tokyo, Japan) in the range between 180 and 450. Distilled water was used as the reference.

2.7. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The free radical scavenging activity was estimated according to the method of Blois (1958). One milliliter of sample (4 mg/mL) was added to 0.2 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical solution (Sigma–Aldrich Co., St. Louis, MO, 1 mL) and distilled water (1 mL) for a blank. The mixture was shaken and left to stand for 30 min at room temperature and measured at 517 nm with a spectrophotometer (UV-1601PC, Shimadzu Co.). The DPPH radical scavenging activity was estimated from the difference in the absorbances for the samples and the blank and expressed as a percentage of DPPH scavenging.

2.8. Reducing power

The reducing power of HA was determined according to the method of Oyaizu (1986). One milliliter of HA (4 mg/mL) was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide ($K_3Fe(CN_6)$). The reaction mixture was incubated in a temperature-controlled water bath at 50 °C for 20 min, followed by addition of 2.5 mL of 10% trichloroacetic acid. The mixture was then centrifuged at 750*g* using a centrifuge (VS-5500, Vision Scientific Co. Ltd., Seoul, Republic of Korea) for 5 min at 25 °C. The supernatant obtained (5 mL) was treated with 5 mL of distilled water and 1 mL of 1% $FeCl_3$. The absorbance

of the reaction mixture was measured as 700 nm. Increase in absorbance was used as the measure of reducing power.

2.9. Statistical analysis

All of the experiments were carried out in triplicate. The data was analyzed with SAS software (V. 8.02, SAS Institute, Cary, NC). The general linear model procedure was processed and Duncan's multiple range test was used to compare the mean values at $P < 0.05$. Mean values and pooled standard errors of the mean (SEM) were recorded.

3. Results and discussion

3.1. Degradation of hyaluronic acid in the powder

This study was designed to degrade high molecular weight hyaluronic acid (HMW-HA) in powder form by different methods including electron beam irradiation (EB), gamma ray irradiation (GM), microwave irradiation (MW), and thermal treatment (TH). The targeted molecular weight of LMW-HA was in the range of 200–230 kDa used in biological activities and industrial applications (Kim et al., 2008a; Lapčík et al., 1998).

To degrade HMW-HA in powder form, the conditions necessary for degradation such as irradiation dose (kGy) for EB and GM or degradation time for MW and TH were first selected. EB- and GM treatment at the absorbed dose of 50 kGy produced LMW-HA with the targeted molecular weight (Table 1). MW treatment for 13 h degraded HMW-HA to LMW-HA (223 kDa), and TH treatment for 52 h at 90 °C degraded it to 229 kDa of LMW-HA.

In this study, ultrasonication (US) and acidic degradation (AC) were also tested, but the following problems precluded the use of these two methods: with US, HMW-HA powder was not degraded to the targeted molecular size (200–230 kDa). Furthermore, polydispersity gradually increased (data not shown), indicating that LMW-HA with a very broad range of molecular mass could be produced and it was felt that these problems might limit the use of the US method in industrial applications. In acidic degradation, when the mixture of acid in ethanol (ethanol + HCl, pH 2) was added to HMW-HA powder, HA was degraded. But, NaOH could not be used for neutralization because of production of water. For this reason, absolute ethanol washing was tried for removing residual HCl in the degrading solution, but the degradation process could not be halted.

Polydispersity (Mw/Mn) of LMW-HA was found to decrease after all of the degradation treatment (Table 1) was completed. Polydispersity is assumed to provide information about the degradation conditions of polymers. When the polydispersity of polymer is initially greater than 2, it would eventually reduce to 2 with progress of random chain scission (Vodeničarová, Dřímalová, Hromádková, Malovíková, & Ebringerová, 2006). EB- and GM-treated LMW-HA showed the lowest polydispersity, indicating that LMW-HA with a narrow molecular distribution could be obtained with these degradation methods. This is the advantage available with EB and GM when they are applied to manufacture LMW-HA in the industrial field. Meanwhile, LMW-HA with MW produced a higher value of polydispersity. These findings showed that the EB and GM methods degraded HMW-HA powder more randomly than the microwave method.

The Hunter color values of LMW-HA obtained with the different degradation methods are also shown in Table 1. MW-treated LMW-HA showed higher b* (yellowness) and a* (redness) but lower L* (brightness) values than those of control, eventually becoming brown in color. TH-treated LMW-HA also showed a significantly higher b* value than that of HMW-HA. These changes in color might limit the application of LMW-HA in cosmetics or food ingredient industries. However, EB- and GM-treated LMW-HAs showed little color change by degradation. The color change in LMW-HA by degradation was due to the changes in the main structure of LMW-HA like double-bond formation by chain scission (Nagasawa, Mitomo, Yoshii, & Kume, 2000). Otherwise, the prolonged time at high temperature needed for degradation with the MW- and TH treatments could turn polymers yellowish because of the oxidation that occurs under aerobic conditions.

3.2. FT-IR spectroscopy

Fig. 1 shows the FT-IR spectra in the spectral range from 2000 to 800 cm^{-1} for LMW-HAs obtained with different degradation methods and HMW-HA. For control, the main bands indicate a C=O stretching at 1653 and 1617 cm^{-1} corresponding to the amide I and acid groups, respectively. The NH group at 1563 and 1320 cm^{-1} (amide II and III), C—O group at 1411 cm^{-1} (acid), C—O—C group at 1150 cm^{-1} (O-bridge), C—O (exocyclic), and C—C groups at 1079 cm^{-1}, and C—OH group at 1042 cm^{-1} are also shown (Berriaud, Milas, & Rinaudo, 1998). The FT-IR spectra of the HMW-HA and the degraded LMW-HA powders (Fig. 1) showed no substantial changes in the spectral pattern in the vicinity of the absorption bands corresponding to vibrations of the acetamido- and carboxylate groups and the pyranose ring (Gilli, Kačuráková, Mathlouti, Navarini, & Paoletti, 1994), even in MW-treated LMW-HA with which significant changes in color occurred. In the previous study which used HA solutions (4 mg/mL) for degradation by gamma ray irradiation, differences in the height and shape of certain absorption bands at 1700–1750 cm^{-1} were seen, which is a phenomenon associated with the formation of carboxylic acid (Hayes et al., 1995). Bezáková et al. (2008) reported that microwave-irradiated HA solutions (1 mg/mL) showed a narrowing of the original broad asymmetric band at 3450–3100 cm^{-1}, corresponding to stretching vibration of the hydroxyl groups after a degradation time of >60 min, which is an indication of a reduction in hydrogen-bond strength. But, in this study, degraded LMW-HA with 200–230 kDa size in a powder form maintained the fundamental structure of the HMW-HA after depolymerisation, implying a weak side-reaction condition in non-solvent systems and a direct degradation effect of the applied energies.

Table 1
Average molecular weight and Hunter color values of hyaluronic acid powder.

	Mw (kDa)	Mw/Mn	Hunter color values		
			L*-value (brightness)	a*-value (redness)	b*-value (yellowness)
Control	1042^{a}	3.52^{a}	90.45^{a}	0.006^{b}	1.363^{e}
EB	205^{b}	2.21^{c}	90.07^{b}	-0.328^{d}	2.487^{c}
GM	211^{b}	2.27^{c}	88.91^{c}	-0.246^{c}	2.274^{d}
MW	223^{b}	2.61^{ab}	87.60^{d}	0.053^{a}	11.250^{a}
TH	229^{b}	2.39^{b}	90.30^{a}	-0.206^{c}	5.201^{b}

* Values with different letters (a–e) in the same column differ significantly ($P < 0.05$).

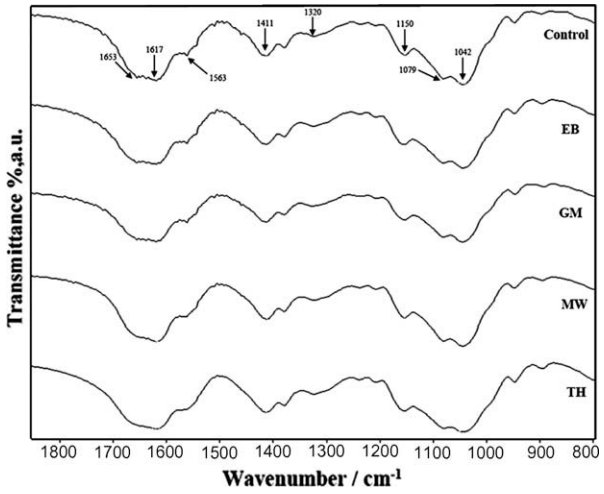

Fig. 1. FT-IR spectra of intact HA and low molecular HAs degraded by each of the four methods.

3.3. UV spectrum

The molecular characteristics of the degraded LMW-HA were monitored by UV spectrometry (Fig. 2). The absorbance at 265 nm was attributed to the double bonds of HA formed after main chain scission and/or hydrogen abstraction reaction after degradation (Nagasawa et al., 2000). Ulanski and Rosiak (1992) reported that the formation of peaks between 250 and 280 nm in chitosan was due to the carbonyl- and carboxyl groups. EB- and GM-treated LMW-HA showed the lowest absorbance changes comparable to those seen with HMW-HA. This observation shows that the fundamental structure of the HMW-HA was well preserved during the course of these degradation methods. In contrast, LMW-HA degraded by MW revealed an increased absorbance in all spectra compared to that of other samples (Fig. 2), which suggested greater formation of carbonyl- or carboxyl groups. Bezáková et al. (2008) also reported that the absorbance at ~210 nm attributed to carboxyl groups increased with increasing microwave time with HA solutions, and that an expanding absorption band at ~240 nm appeared after 180 min of MW treatment indicating the presence of unsaturated structures, such as the 4,5-unsaturated glucuronic acid moieties absorbing (Uchiyama, Dobashi, Ohkouchi, & Nagasawa, 1990). TH-treated LMW-HA showed higher UV absorbances than EB- and GM-treated ones, but lower than the MW-treated. The high polydispersity of MW-treated LMW-HA could be related to the formation of large amounts of the carbonyl group. For polymers with the same average molecular weight, a higher polydispersity means a broader molecular weight distribution, with a higher number of small fragments. When the number of small molecules is higher, the number of newly formed double bonds could increase. Therefore, the MW-treated LMW-HA with high polydispersity could have formed more double bonds.

In Fig. 2, UV spectrum of MW-treated LMW-HA considerably changed from original one. While in Fig. 1, FT-IR spectrum of MW-treated LMW-HA was the same to HMW-HA. This was because the primary structure of HA was not changed by MW treatment and there were already the peaks representing double bonds of carbonyl and carboxyl groups in FT-IR spectrum of HMW-HA. Bezáková et al. (2008) and Dřímalová, Velebný, Sasinková, Hromádková, and Ebringerová (2005) have also reported that in the UV spectra of the HA degraded by microwave irradiation the absorbances at ~210 and ~232 nm were increased by the formation of double bonds. But, it was not evidenced in FT-IR ad NMR spectra because the primary structure was maintained.

According to the results of both FT-IR and UV, the degradation of HA by these described methods caused the main chain of HA to break at glycosidic linkages, which later led to the formation of carbonyl- and carboxyl groups.

3.4. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability

The DPPH radical scavenging ability of HA is illustrated in Fig. 3. All of the LMW-HA displayed an increased DPPH radical scavenging ability after degradation, especially MW-treated LMW-HA. Alkrad, Mrestani, Stroehl, Wartewig, and Neubert (2003) reported that when HA was digested with the enzyme hyaluronidase, a double bond was formed and this double bond was essential for reducing toxicity of the radicals ($ROO^{\cdot}$, $HO^{\cdot}$). Alternatively, another antioxidant mechanism might produce the direct scavenging effect of HA on free radical molecules, especially the detrimental $OH^{\cdot}$ or other Fenton's reaction intermediates like $O_2^{\cdot-}$ (Campo et al., 2004). The highest DPPH radical scavenging activity of MW-treated LMW-HA might be correlated with the highest UV absorption in 265 nm, which shows double-bond formation in LMW-HA. The browning of MW-treated LMW-HA may also be due to a double-bond formation and this could lead to an increase in the DPPH radical scavenging activity (Nagasawa et al., 2000). Increase in the DPPH scavenging activity of GM-treated LMW-HA correlates with

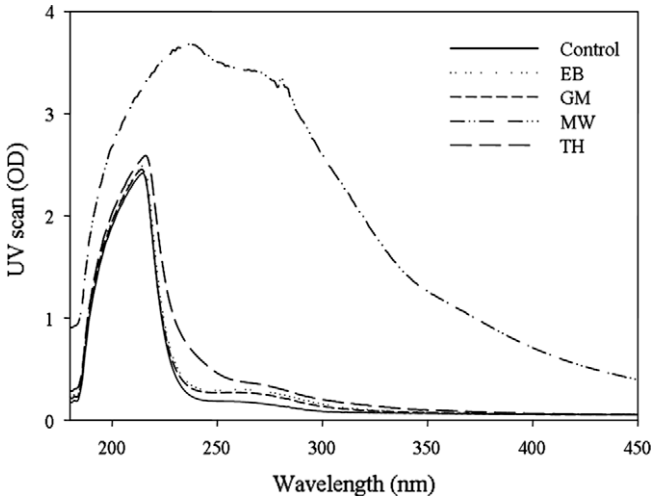

Fig. 2. UV spectra of intact HA and low molecular HAs degraded by each of the four methods.

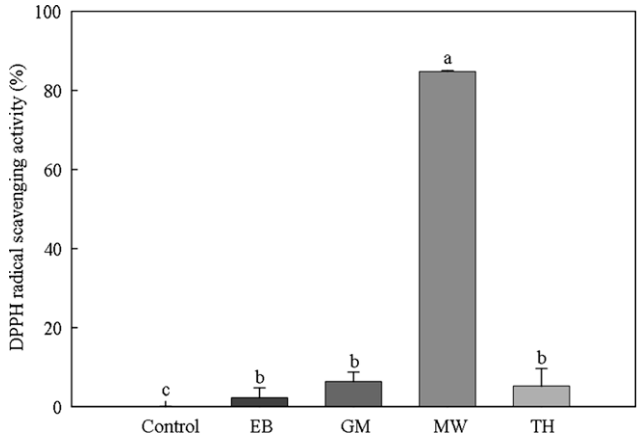

Fig. 3. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of intact HA and low molecular HAs degraded by each of the four methods. Alphabets (a, b, c) mean significant difference at $P \leqslant 0.05$.

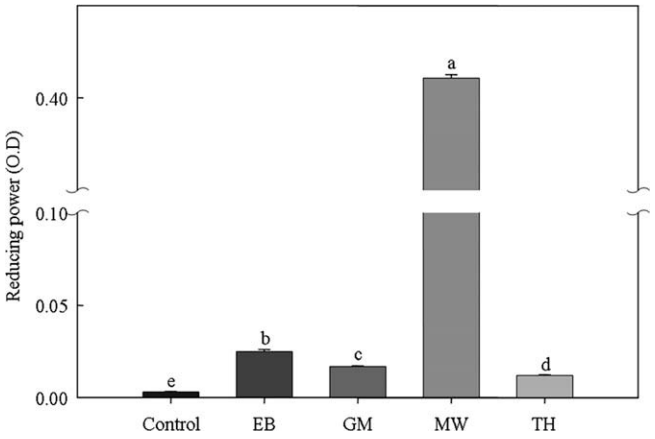

Fig. 4. Reducing power of intact HA and low molecular HAs degraded by each of the four methods. Alphabets (a, b, c, d, e) point to significant differences at $P \leqslant 0.05$.

the findings in previous studies by Kim et al. (2008b). The DPPH scavenging ability of gamma-irradiated HA solution increased depending on the absorbed dose, which is correlated with increasing UV absorption in 265 nm. However, GM-, EB-, and TH-treated LMW-HA powders showed lower DPPH radical scavenging activities than MW-treated LMW-HA, and this might be the reason for the smaller increase in UV absorbance after degradation encountered in previous studies.

3.5. Reducing power

In this assay, the yellow color of the test solution changed to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (i.e. antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can be useful in monitoring the Fe^{2+} concentration.

Fig. 4 shows the comparisons of reducing power after HMW-HA was degraded by the different degradation methods. MW-treated LMW-HA powder showed the greatest reducing power, which is in keeping with the DPPH radical scavenging activity. EB-, GM-, and TH-treated LMW-HA also showed improved reducing power over HMW-HA. Reducing capacity is generally associated with hydrogen-donating ability (Shimada, Fujikawa, Yahara, & Nakamura, 1992). Thus, reducing capacity of a compound could serve as a significant indicator of its antioxidant activity potential. The antioxidant activity of a compound has been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, reductive capacity, and radical scavenging. The general antioxidant mechanism of HA is known to be its carboxylic group (Campo et al., 2004). This charged group may interact with transition metal ions like Cu^{2+} or Fe^{2+} that are in turn responsible for the initiation of Fenton's reaction. The ability of HA to chelate different ions and transition metals has been extensively reported by several authors (Merce, Carrera, Romanholi, & Recio, 2002; Nagy et al., 1998). Therefore, increase in reducing power of MW-treated LMW-HA may be also correlated with the UV spectrum and the Hunter color value results that indicate the formation of double bonds by chain scission.

4. Conclusion

Several methods have been employed to degrade high molecular weight hyaluronic acid into lower molecular mass fragments, because many studies have investigated the advantages of lower molecular weight of HA for certain applications in the fields of medical treatment and cosmetics. This study compared four different methods of degradation of HA powder and the results revealed that all the tested treatments not only reduce the molecular mass but also modify the structure of the initial polymer to various extents, depending on the method employed. Especially, microwave irradiation increased UV absorbance at 265 nm and changed the color of the LMW-HA to brown, which might bring about an increase in antioxidant activities. Electron beam- and gamma ray irradiation resulted in low polydispersity of degraded HA, and minimized the change in UV spectra compared to other methods, which indicates little change occurred in molecular structure during degradation. These results will be helpful in producing LMW-HA with the superior biological activity needed for industrial applications.

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Reference

Alkrad, J. A., Mrestani, Y., Stroehl, D., Wartewig, S., & Neubert, R. (2003). Characterization of enzymatically digested hyaluronic acid using NMR, Raman, IR, and UV–vis spectroscopies. *Journal of Pharmaceutical and Biomedical Analysis, 31*, 545–550.

Berriaud, N., Milas, M., & Rinaudo, M. (1998). *Polysaccharides: Structural diversity and functional versatility*. New York; Marcel Dekker.

Bezáková, Z., Hermannová, M., Drímalová, E., Malovíková, A., Ebringerová, A., & Velebný, V. (2008). Effect of microwave irradiation on the molecular and structural properties of hyaluronan. *Carbohydrate Polymers, 73*, 640–646.

Blois, M. S. (1958). Antioxidant determination by the use of a stable free radical. *Nature, 181*, 1199–1200.

Bothner, H., Waaler, T., & Wik, O. (1998). Limiting viscosity number and weight average molecular weight of hyaluronate samples produced by heat degradation. *International Journal of Biological Macromolecules, 10*, 287–297.

Campo, G. M., Avenoso, A., Campo, S., D'Ascola, A., Ferlazzo, A. M., & Calatroni, A. (2004). The antioxidant and antifibrogenic effects of the glycosaminoglycans hyaluronic acid and chondroitin-4-sulphate in a subchronic rat model of carbon tetrachloride-induced liver fibrogenesis. *Chemico-Biological Interactions, 148*, 125–138.

Dřímalová, E., Velebný, V., Sasinková, V., Hromádková, Z., & Ebringerová, A. (2005). Degradation of hyaluronan by ultrasonication in comparison to microwave and conventional heating. *Carbohydrate Polymers, 61*, 420–426.

Fitzgerald, K. A., Bowie, A. G., Skeffington, B. S., & O'Neill, L. A. (2000). Ras, protein kinase C zeta, and I kappa B kinases 1 and 2 are downstream effectors of CD44 during the activation of NF-kappa B by hyaluronic acid fragments in T-24 carcinoma cells. *Journal of Immunology, 164*, 2053–2063.

Fraser, J. R., Laurent, T. C., & Laurent, U. B. (1997). Hyaluronan: Its nature, distribution, functions and turnover. *Journal of Internal Medicine, 24*, 27–33.

Gilli, R., Kačuráková, M., Mathlouti, M., Navarini, L., & Paoletti, S. (1994). FTIR studies of sodium hyaluronate and its oligomers in the amorphous solid phase and in aqueous solution. *Carbohydrate Research, 263*, 315–326.

Goa, K. L., & Benfield, P. (1994). Hyaluronic acid: A review of its pharmacology and use as a surgical aid in ophthalmology, and its therapeutic potential in joint decease and wound-healing. *Drugs, 47*, 536–566.

Gura, E., Hückel, M., & Müller, P. J. (1998). Specific degradation of hyaluronic acid and its rheological properties. *Polymer Degradation and Stability, 59*, 297–302.

Hayes, D. J., Murano, E. A., Murano, P. S., Olson, D. G., & Sapp, S. G. (1995). *Food irradiation: A source book*. Iowa: Iowa State University Press.

Hokputsa, S., Jumel, K., Alexander, C., & Harding, S. E. (2003). A comparison of molecular mass determination of hyaluronic acid using SEC/MALLS and sedimentation equilibrium. *European Biophysics Journal, 32*, 450–456.

Horton, M. R., Shapiro, S., Bao, C., Lowenstein, C. J., & Noble, P. W. (1999). Induction and regulation of macrophage metalloelastase by hyaluronan fragments in mouse macrophages. *Journal of Immunology, 162*, 4171–4176.

Kim, J. K., Sung, N. Y., Srinivasan, P., Choi, J. I., Kim, S. K., Oh, J. M., et al. (2008a). Effect of gamma irradiated hyaluronic acid on acetaminophen induced acute hepatotoxicity. *Chemico-Biological Interactions, 172*, 141–153.

Kim, J. K., Srinivasan, P., Kim, J. H., Choi, J. I., Park, H. J., Byun, M. W., et al. (2008b). Structural and antioxidant properties of gamma irradiated hyaluronic acid. *Food Chemistry, 109*, 763–770.

Kubo, K., Nakamura, T., Takagaki, K., Yoshida, Y., & Endo, M. (1993). Depolymerisation of hyaluronan by sonication. *Glycoconjugate Journal, 10*, 435–439.

Lapčík, L., Jr., Lapčík, L., De Smedt, S., Demeester, J., & Chrabreček, P. (1998). Hyaluronan: Preparation, structure, properties, and applications. *Chemical Reviews, 98*, 2663–2684.

Mason, T. J., & Lorimer, J. P. (2002). *Applied sonochemistry: Uses of ultrasound in chemistry and processing*. New York: Wiley.

McKee, C. M., Penno, M. B., Cowman, M., Burdick, M. D., Strieter, R. M., Bao, C., et al. (1996). Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. *Journal of Clinical Investigation, 98*, 2403–2413.

Merce, A. L. R., Carrera, L. C. M., Romanholi, L. K. S., & Recio, M. A. L. (2002). Aqueous and solid complexes of iron(III) with hyaluronic acid: Potentiometric titrations and infrared spectroscopy studies. *Journal of Inorganic Biochemistry, 89*, 212–218.

Miyazaki, T., Yomota, C., & Okada, S. (2001). Ultrasonic depolymerization of hyaluronic acid. *Polymer Degradation and Stability, 74*, 77–85.

Nagasawa, N., Mitomo, M., Yoshii, F., & Kume, T. (2000). Radiation-induced degradation of sodium alginate. *Polymer Degradation and Stability, 69*, 279–285.

Nagy, L., Yamashita, S., Yamaguchi, T., Sipos, P., Wakita, H., & Nomura, M. (1998). The local structure of Cu(II) and Zn(II) complexes of hyaluronate. *Journal of Inorganic Biochemistry, 72*, 49–55.

Noble, P. W., McKee, C. M., Cowman, M., & Shin, H. S. (1996). Hyaluronan fragments activate an NF-kappa B/I-kappa B alpha autoregulatory loop in murine macrophages. *Journal of Experimental Medicine, 183*, 2373–2378.

Oertli, B., Beck-Schimmer, B., Fan, X., & Wuthrich, R. P. (1998). Mechanisms of hyaluronan-induced up-regulation of ICAM-1 and VCAM-1 expression by murine kidney tubular epithelial cells: Hyaluronan triggers cell adhesion molecule expression through a mechanism involving activation of nuclear factor-kappa B and activating protein-1. *Journal of Immunology, 161*, 3431–3437.

Ohkawara, Y., Tamura, G., Iwasaki, T., Tanaka, A., Kikuchi, T., & Shirato, K. (2000). Activation and transforming growth factor-beta production in eosinophils by hyaluronan. *American Journal of Respiratory Cell and Molecular Biology, 23*, 444–451.

Orviský, E., Šoltés, L., Chabreček, P., Novák, I., & Stančiková, M. (1993). Size exclusion chromatographic characterization of sodium hyaluronate fractions prepared by high energetic sonication. *Chromatographia, 37*, 20–22.

Oyaizu, M. (1986). Antioxidant activity of browning products of glucosamine fractionated by organic solvent and thin-layer chromatography. *Nippon Shokuhin Kogyo Gakkaishi, 35*, 771–775.

Shimada, K., Fujikawa, K., Yahara, K., & Nakamura, T. (1992). Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry, 40*, 945–948.

Tokita, Y., & Okamoto, A. (1995). Hydrolytic degradation of hyaluronic acid. *Polymer Degradation and Stability, 48*, 269–273.

Uchiyama, H., Dobashi, Y., Ohkouchi, K., & Nagasawa, K. (1990). Chemical change involved in the oxidative reductive depolymerization of hyaluronic acid. *Journal of Biological Chemistry, 265*, 7753–7759.

Ulanski, P., & Rosiak, J. M. (1992). Preliminary study on radiation-induced changes in chitosan. *Radiation Physics and Chemistry, 39*, 53–57.

Vodeničarová, M., Dřímalová, G., Hromádková, Z., Malovíková, A., & Ebringerová, A. (2006). Xyloglucan degradation using different radiation sources: A comparative study. *Ultrasonics Sonochemistry, 13*, 157–164.

West, D. C., Hampson, I. N., Arnold, F., & Kumar, S. (1985). Angiogenesis induced by degradation products of hyaluronic acid. *Science, 228*, 1324–1326.

Zeng, C., Toole, B. P., Kinney, S. D., Kuo, J. W., & Stamenkovic, I. (1998). Inhibition of tumor growth *in vivo* by hyaluronan oligomers. *International Journal of Cancer, 77*, 396–401.