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Structural and antioxidant properties of gamma irradiated hyaluronic acid

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Abstract

Hyaluronic acid (Hyaluronan, HA) was depolymerised by gamma irradiation and its structural changes and antioxidant activities were investigated. The structural changes of gamma irradiated HA were studied by gel-permeation chromatography (GPC), viscosity, pH, Hunter colour measurement, UV spectrophotometry, and FT-IR spectroscopy. The results demonstrated that gamma irradiation decreased molecular weight size, viscosity and pH of the hyaluronic acid and its colour turned to intense yellow. UV spectra of the irradiated HA showed a change at 265 nm, which indicates the formation of double bonds. Differences in the height and shape of certain absorption bonds of FT-IR spectra in the range 1700–1750 cm^{-1} were also observed, which is associated with the formation of carboxylic acid. From these structural changes of the HA, gamma irradiation may have a role in the formation of pyrancarboxylic acid rings. DPPH radical scavenging ability and the reducing power of gamma irradiated HA were significantly higher than that of non-irradiated HA. However, non-irradiated and irradiated HA did not show significant differences in the Rancimat test.

Keywords: Hyaluronic acid; Gamma irradiation; Depolymerisation; Antioxidant activity

1. Introduction

Hyaluronic acid (also called Hyaluronan, Hyaluronate, or HA) is a linear polyelectrolyte based on β1-4-D-glucuronic acid (GlcA) and β1-3 *N*-acetyl-D-glucosamine (GlcNAc) repeat units. The high molecular mass HA, which plays an important role in many biological processes such as in tissue hydration, proteoglycan organization in the extracellular matrix, and tissue repair, has found application in several clinical treatments and cosmetic uses (Arshinoff, 1995; Goa & Benfield, 1994; Lapčík, Lapčík, De Smedt, Demeester, & Chrabreček, 1998; Laurent, 1998). In recent years, the low molecular mass HA has also been reported to aid wound healing by promoting angiogenesis (West, Hampson, Arnold, & Kumar, 1985). HA fragments can also, under specific circumstances, promote early inflammation, which is critical to initiate wound healing, which can then modulate later stages of the process, allowing for matrix stabilization and reduction of long-term inflammation (Morra, 2005). Functional maturation of dendritic cells during inflammation, inhibition of anchorage-independent growth of tumour cells and protection of granulation tissue from oxygen free radical damage have also been ascribed to a mixture of hyaluronic acid fragments (Ghatak, Misra, & Toole, 2002; Termeer et al., 2000; Trabucchi et al., 2002). Furthermore, it was also reported that enzymatically digested HA revealed a better capacity as a radical scavenger than intact HA (Gerlach, 2000).

Many methods have been applied to depolymerise HA into lower molecular mass fragments such as ultrasonication

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(Mason & Lorimer, 2002), oxidative degradation (Hokputsa, Jumel, Alexander, & Harding, 2003), high temperature in an autoclave (Bothner, Waaler, & Wik, 1998), acid hydrolysis (Tokita & Okamoto, 1995) and microwave irradiation (Gelema, 1997). Depolymerisation of HA was also achieved by enzymatic, chemical or physical means. Enzymatic methods for degrading hyaluronic acid are also known, but they are relatively uncontrollable and tend to broaden the hyaluronic acid molecular weight distribution and therefore increase the polydispersity of the material. This makes a material particularly unsuitable for certain applications where highly defined molecular weight ranges are required. Chemical methods suffer similar problems and, moreover, may result in residual concentrations of the reacting chemicals remaining in a therapeutic product. Fractionation of hyaluronic acid into defined molecular weight species is feasible, but this is a complex operation which is not easily controllable for large scale manufacturing operations.

Gamma irradiation has been found to be effective for depolymerisation of cellulose (Charlesby, 1982), starch (Sokhey & Chinnaswamy, 1992), chitosan (Choi, Ahn, Lee, Byun, & Park, 2002), and pectin (Zegota, 1999). Cleavage of the molecular chain is ascribed (Sohma, 1983) to decaying processes of free radicals generated at the primary stage of gamma irradiation. Due to macroradical formation and their further reactions, both depolymerisation methods are accompanied to various extents with changes in chemical composition and primary structure of the polysaccharides. In addition, many studies have shown that gamma irradiation considerably affected the antioxidant properties of truffles (Adamo et al., 2004), ground beef (Ahn & Nam, 2004), herbs and spices (Calucci et al., 2003), green tea leaf extracts (Jo, Son, Lee, & Byun, 2003), lupin seed products (Lampart-Szczapa, Korczak, Nogala-Kalucka, & Zawirska-Wojtasiak, 2003), and soybean (Variyar, Limaye, & Sharma, 2004). However, the effect of gamma irradiation on hyaluronic acid has not been investigated.

The main purpose of the present investigation is to study the structural changes of gamma irradiated HA with several experiments such as gel-permeation chromatography (GPC), viscosity, pH, Hunter colour measurement, UV spectrophotometry, and FT-IR spectroscopy and to elucidate the antioxidant activities of hyaluronic acid treated with various doses of gamma irradiation.

2. Materials and methods

2.1. Sample preparation

Hyaluronic acid powder isolated from *Streptococcus zooepidecus* was purchased from KOLON Life Science, Inc. (Kyung ki-Do, Korea) and dissolved in distilled water as 0.4% w/v.

The solutions in tightly capped tubes were irradiated by a cobalt-60 irradiator (point source, AECL, IR-79, Nordion, Canada) with 0, 1, 3, 5, 10, and 50 kGy. The source strength was approximately 100 kCi with a dose rate at the location of the sample of approximately 70 Gy/min. Irradiation was carried out at 15 ± 0.5 °C. Dosimetry was performed by using alanine dosimeters (Bruker Instruments, Rheinstetten, Germany) measured with a Bruker EMS 104 EPR Analyzer. The alanine pellet was attached to the tube. The actual doses were within ±2% of the target dose. The containers were continuously rotated during an irradiation to obtain a uniform dose. The samples were kept at 4 °C in a refrigerator prior to the following experiments.

2.2. Structural properties

2.2.1. Gel-permeation chromatography (GPC)

GPC was performed using a Waters 2690 separation module (Waters Co., Milford, MA, USA), a refractive index detector (RI, Waters 2410, Waters Co.), Empower software (System Software, Empower option GPC, Waters Co.), and a PL aquagel-OH, −60, −40, and −30 column (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 a pullulan standard (Showa Denko K.K., Japan).

2.2.2. Viscosity

The viscosities of all samples were measured using a Brookfield Model DV-II+Pro viscometer (Brookfield Engineering Laboratories, Inc., MA, USA) with spindle No. 2 at 75 rpm. Samples were added to the 250 ml flask and allowed to equilibrate for at least 2 min prior to testing at ambient temperature. Each determination of viscosity (cP) was done in triplicate.

2.2.3. pH

The pH of the samples was measured using a portable pH meter (Orion 520A, Orion Research Inc., Boston, MA, USA), which was calibrated with pH 7 and pH 4 buffer.

2.2.4. Hunter colour measurement

The HA solutions were transferred into a glass cell (CM A-98, 10 mm in width) and measured with a Colour Difference Meter (Spectrophotometer CM-3500d, Minolta Co., Ltd. Osaka, Japan). The instrument was calibrated to standard black and white tiles before an analysis. A large size aperture was used and the measurements were done in triplicate.

2.2.5. UV spectrophotometry

UV–visible spectroscopy of irradiated HA solutions was carried out at 25 °C using a spectrophotometer (model UV-1601PC, Shimadzu Co.) in the range of 180–450 nm. Distilled water was used as a reference.

2.2.6. FT-IR spectroscopy

The Fourier-transform infrared (FT-IR) spectra were acquired by using a Bruker Spectrometer VERTEX 70 at the wavelength region between 200 and 400 cm^{-1}. Samples were prepared as a thin film of the HA mixed with KBr at polymer/KBr w/w ratio 1–100. Obtained spectra were the result of 24 scans at the spectrophotometer resolution 8 cm^{-1}.

2.3. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity

The free radical scavenging effect was estimated according to the method of Blois (1958) with some modifications. One milliliter of HA (4 mg/ml) were added into the 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Sigma–Aldrich Co., St. Louis, MO, USA, 1 ml) and 95% ethanol (1 ml) for a sample blank. The mixture was shaken and left to stand for 30 min at room temperature and measured at 517 nm with a spectrophotometer (model UV-1601PC, Shimadzu Co.). The DPPH radical scavenging capacity was estimated from the difference in the absorbance for the samples or sample blank and expressed as a percentage of the DPPH scavenging.

2.4. Reducing power

The reducing power of HA was determined according to the method of Oyaizu (1986) with a slight modification. 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 mixtures were 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 mixtures were then centrifuged at 750 g using a centrifuge (VS-5500, Vision scientific Co. Ltd., Seoul, 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 at 700 nm. An increase in absorbance was used as a measure of the reducing power.

2.5. Rancimat test

Measurement of the antioxidant potency of HA in a food matrix (corn oil) was performed using Metrohm Rancimat Model 743 (Herisan, Switzerland). The Rancimat apparatus was operated at 120 °C. A dry air flow of 20 l/h was passed through the oil sample (4 ± 0.001 g) containing the antioxidant (1 ± 0.001 g) with tween 80 (0.1 ± 0.001 g) as a surfactant. The volatile oxidation products come from the oxidation of the oil dissolved in cold milli-Q water (50 ml), causing an increase in the electrical conductivity. The time (in h) taken to reach a specific conductivity value, corresponding to the flex point of the peroxidation curve, was considered as the induction period (IP). The effect of the samples on retarding the corn oil oxidation, interpreted as the protection factor (Pf), was calculated according to the following expression:

$$Pf = IP_{antiox.}/IP_{contrl.}$$

The higher the Pf was the higher the antioxidant potency of the compounds. All tests were performed in triplicate.

2.6. Statistical analysis

The data was analyzed by SAS software (V. 8.02, SAS Institute, Cary, NC, USA). The general linear model procedure was processed and the 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. Structural changes of gamma irradiated hyaluronic acid

Gamma irradiation produced a significant reduction in the Mw of hyaluronic acid (HA) as shown in Table 1. As can be seen, the depolymerisation proceeded rapidly at a low dose (1 kGy) and further increasing dose also significantly decreased the Mw of HA. These results are due to the presumed mechanism of a cleavage of the glycosidic bonds between β1-4-D-glucuronic acid (GlcA) and β1-3 *N*-acetyl-D-glucosamine (GlcNAc) repeat units (Fuchs & Heusinger, 1994; Sultanov & Turaev, 1996). The H· and ·OH radicals formed by radiolysis during irradiation of water accelerated the molecular chain scission of HA. Reaction between the above free radicals and HA molecules leads to rapid degradation of HA in an aqueous solution. When the irradiation was applied directly to the HA in the solid phase, the Mw also decreased linearly with the dose of gamma irradiation from 2 to 90 kGy (Miller & Shiedlin, 2002). However, Reháková, Bakoš, Soldán, and Vizárová (1994) reported that high-Mw products from a radical recombination were observed by gamma irradiation of solution and powder HA with 6 kGy. The free radicals formed by radiolysis of water are even effective in enhancing crosslinking of HA. Therefore the optimum target dose should be determined before any application because irradiation of natural polymers can result in both degraded and cross-linked polymers (Kume, Nagasawa, & Yoshii, 2002).

The viscosity of HA was dramatically decreased by gamma irradiation with 1 kGy (Table 1). Practically, there was an obvious difference in the viscosity of HA with and without irradiation; gamma irradiation reduced the viscosity of HA as was also observed in previous studies (Lurie, Offer, Russo, Samuni, & Nitzan, 2003).

Gamma irradiation significantly decreased the pH of HA (Table 1). Increased acidity of irradiated HA could be due to a breakdown of HA molecules, and it might be due to the formation of carboxylic groups. Sokhey and Chinnaswamy (1993) reported similar results, indicating

Table 1
Physical changes of gamma irradiated hyaluronic acid (HA)[A]

IR dose (kGy)	Mw (kDa)	Viscosity (cP)	pH	Hunter colour values		
				L^*-value	a^*-value	b^*-value
0	1110.14[a]	499.25[a]	6.09[a]	97.71[d]	0.008[a]	0.053[e]
1	154.97[b]	18.00[b]	5.49[b]	99.83[ab]	−0.027[b]	0.048[e]
3	67.81[c]	13.00[c]	5.44[c]	99.84[ab]	−0.045[c]	0.093[d]
5	33.72[d]	10.50[e]	5.43[c]	99.85[a]	−0.077[d]	0.171[c]
10	26.77[de]	11.00[d]	5.37[d]	99.82[b]	−0.104[e]	0.279[b]
50	6.52[e]	11.00[d]	5.01[e]	99.49[c]	−0.437[f]	1.136[a]
SEM[B]	6.06	0.10	0.005	0.009	0.005	0.006

[A] Values with different letters (a–f) within the same column differ significantly ($P \leqslant 0.05$).
[B] Standard error of the mean ($n = 18$).

that the breakdown of glycosidic linkages by the action of free radicals may cause an increase in the starch acidity. In addition, in the pH range of HA from 5.0 to 11.0, the intrinsic viscosity [η] was constant, but at a pH less than 5.0, the intrinsic viscosity [η] and molecular radius decreased reversibly due to the protonation of glucuronic acid groups (Gura, Hűckel, & Műller, 1997). In the current investigation, the pH of irradiated HA were above 5.0, thus the decrease in viscosity is not due to decrease in the pH of HA, it is due to depolymerisation of HA, which was found to be in accordance with a previous study by Gura et al. (1997).

The Hunter colour values of the HA with different irradiation doses are shown in Table 1. Gamma irradiated HA showed greater $+b^*$ (more yellow), $+L^*$ (brighter), and $-a^*$ (greener) values, which indicate HA revealed a very intense yellowish colouration. The colour of chitosan solution also changed to a more intense brown with increasing irradiation dose (Choi et al., 2002). Nagasawa, Mitomo, Yoshii, and Kume (2000) investigated the radiation effects on colour change of alginate and concluded that the browning of alginate during radiation was due to a double bond formation by chain scission.

Fig. 1 shows the UV scan spectra of HA irradiated with various doses in a solution form. It revealed a new absorption band at 265 nm and a peak intensity increase with increasing dose. This can be assigned to the double bonds of HA formed after main chain scission and/or hydrogen abstraction reaction by irradiation (Nagasawa et al., 2000). Ulanski and Rosiak (1992) also reported that the formation of peaks between 250 and 280 nm of gamma irradiated chitosan was due to the carbonyl and carboxyl groups.

Fig. 2 shows the FT-IR spectrum in the spectral range 2000–400 cm^{-1} for gamma irradiated HA with 0, 1, 3, 5, 10, and 50 kGy. At 0 kGy, the main bands indicate a C=O stretching at 1653 and 1617 cm^{-1} corresponding to the amide I and acid groups, respectively, NM group at 1563 and 1320 cm^{-1} (amide II and III), C–O group at 1411 cm^{-1} (acid) and C–O–C group at 1150 cm^{-1} (O-bridge), the C–O (exocyclic) and C–C groups at 1079 cm^{-1}, and the C–OH group at 1042 cm^{-1} (Berriaud,

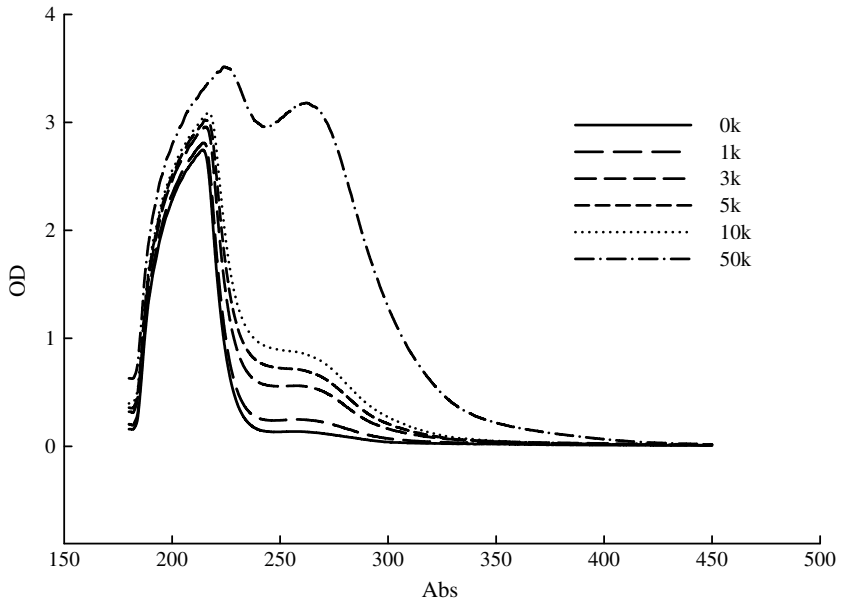

Fig. 1. UV scan spectra of gamma irradiated hyaluronic acid (HA).

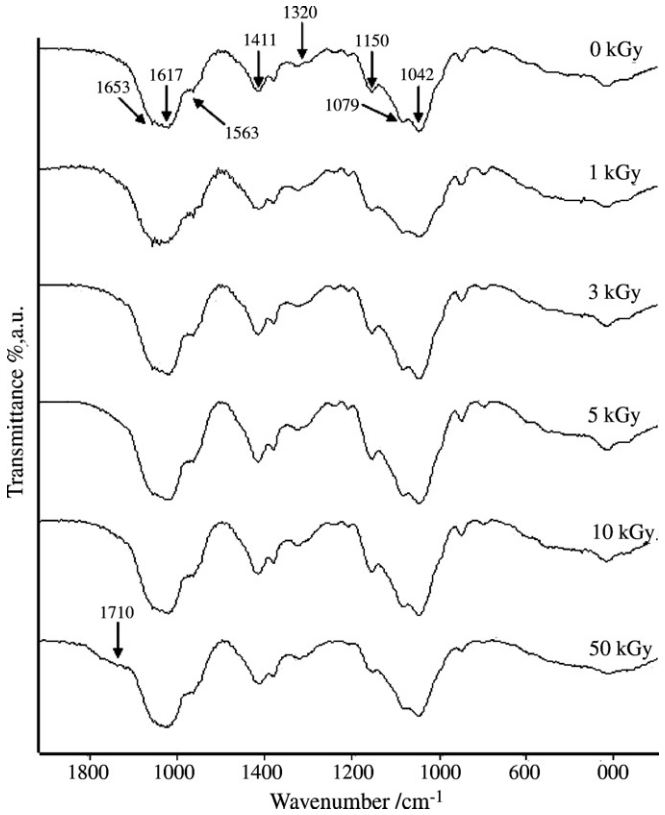

Fig. 2. FT-IR spectra of gamma irradiated hyaluronic acid (HA).

Milas, & Rinaudo, 1998). As can be seen, the overall spectral pattern did not change by decreasing the molecular size of HA and additional bands did not appear even in the spectrum of the most degraded samples. However, there were some differences in the height and shape of certain absorption bands at 1700–1750 cm^{-1}, which is associated with the formation of carboxylic acid. This result correlates well with the results obtained from decreasing pH of HA by gamma irradiation.

In a nutshell, the data presented above may be due to the cleavage of the glycosidic bond in HA and the formation of double bonds and carboxylic acid groups, which correlate well with a previous study by Alkrad, Mrestani, Stroehl, Wartewig, and Neubert (2003) showing the presence of a double bond in the pyrancarboxylic acid ring after the enzymatic digestion of HA. Therefore, gamma irradiation may produce pyrancarboxylic acid rings by structural modification of native HA.

3.2. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability

DPPH radical scavenging ability of HA showed gradual and significant increase ($p \leqslant 0.05$) by increasing dose of gamma irradiation significantly (Fig. 3). Alkrad et al. (2003) reported that when HA was digested with the enzyme hyaluronidase, a double bond in the pyrancarboxylic acid ring was formed. This double bond in the pyrancarboxylic acid ring is necessary for reducing the toxicity

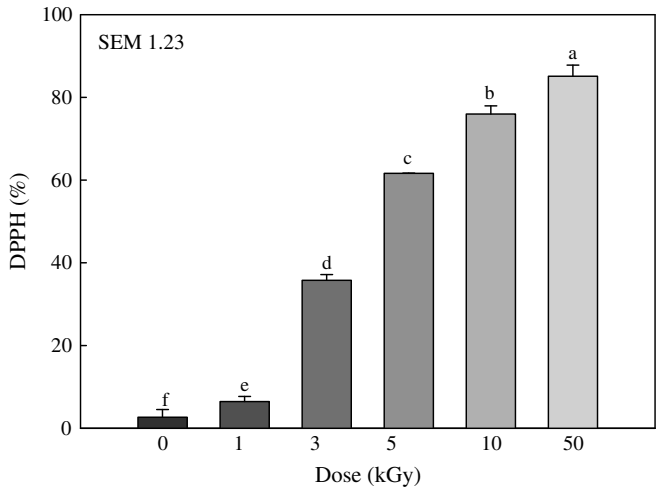

Fig. 3. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effect of gamma irradiated hyaluronic acid (HA). Alphabet (a–f) are significantly different at $p \leqslant 0.05$. Standard error of the mean (SEM, $n = 12$).

of radicals ($ROO^{\cdot}$, $HO^{\cdot}$) as a radical scavenger during UV irradiation of the human skin. The pyrancarboxylic acid ring formation of gamma irradiated HA may also cause increasing DPPH radical scavenging ability which correlates well with the previous data presented above. Alternatively, another antioxidant mechanism may be the directly 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).

Our current investigation shows significant increase in the DPPH scavenging activity which correlates with the earlier studies by Variyar et al. (2004) in which the scavenging ability of soybean, with 0.5–5 kGy of gamma irradiation, on DPPH radicals increased with the doses used. Ahn, Kim, Jo, Kim, and Byun (2004) reported that, after irradiation, a scavenging ability of phytic acid on DPPH radicals was found. However, they pointed out that unirradiated phytic acid did not show scavenging ability. Green tea leaf extracts with 10 and 20 kGy of irradiation significantly increased the scavenging ability on DPPH radicals at 4 °C (Jo et al., 2003).

By contrast, Ahn et al. (2005) found that, immediately after irradiation, the scavenging ability of Chinese cabbage was reduced by 2 kGy of irradiation. No significant changes of the scavenging abilities were observed in unirradiated and 5, 10 and 20 kGy-irradiated *Chungkookjang* and *Doenjang* (Byun, Son, Yook, Lo, & Kim, 2002). Furthermore, electron donating activities of some Korean medicinal herbs were not influenced by 10 kGy of gamma irradiation at an ambient temperature (Byun, Yook, Kim, & Chung, 1999).

3.3. Reducing power

In this assay, the yellow colour of the test solution changes 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 monitor the Fe^{2+} concentration.

Reducing power of HA increased as the gamma irradiation dose increased, as shown by an increase in absorbance at 700 nm ($p \leqslant 0.05$) (Fig. 4). The antioxidant mechanism of HA is due to its carboxylic groups (Campo et al., 2004). This charged group may interact with transition metals 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). Furthermore, solid complexes of Iron(III) with HA are very stable and show binding constants log K of value about 8.0 (Nagy et al., 1998). A species of composition CuL_2, L being a disaccharide of HA, showed a stability constant log K of 3.47 (Lapčík et al., 1998; Pirc, Arcon, Bukovec, & Kodre, 2000). All this data strongly suggest that HA is able to being iron and copper cations in solution, which would decrease their availability for oxidation processes. Therefore, an increase in the reducing power of gamma irradiated HA may due to the formation of a pyrancarboxylic acid ring that may reduce the Fe^{3+}/ferricyanide complex to the ferrous form.

3.4. Antioxidant activity in Rancimat test

The Rancimat test, "accelerated oxidation", is used to obtain information on whether a food (rich in oils or fats) resists heating at a high temperature. Because different substances can accelerate and/or inhibit the formation of hydroperoxides, it is possible to evaluate the protection they offer. The time required for the formation of a sufficient concentration of initiating radicals is greater when samples with antioxidant activity are added, thus delaying the onset of the propagation phase of the radical chain reaction (Martínez-Tomé et al., 2004).

In this experiment, each hyaluronic acid sample was added to corn oil to a final concentration of 0.8 mg/ml. Ascorbic acid was used as a traditional food antioxidant control and a final concentration was 2 mg/ml. Fig. 5 shows the results of the Rancimat test of gamma irradiated HA. As can be seen, 1% of ascorbic acid showed the highest Pf, but irradiation did not affect any significant changes in this test. The antioxidant mechanism of HA is due to its particular chemical structure, which is the presence of carboxylic groups. These charged groups may interact with transition metals ions like Cu^{2+} or Fe^{2+} that are in turn responsible of the initiation of the Fenton's reaction. Transition metal chelation has been classified along with direct radical scavenging, reduction by electron donation and enzymatic antioxidant activity as an important part of antioxidative behavior of compounds (Voegeli, Meier, & Doppler, 1993). In lipid model systems, an iron complexation leads to a lipid protection and a decrease of the amount of lipid peroxidation products (Trommer et al., 2003). This transition metal chelation theory explains the similarity of the protective effects of non-irradiated HA and irradiated HA observed in the Rancimat test. Assuming an iron chelation as the main reason for the antioxidative behavior of HA and transition metal catalysis as the key step in lipid peroxidation the similarity is easily explained. All the samples contain the same or rare concentrations of iron ions and the chelation of them leads to a similar protection from lipid peroxidation as well as a heat induced formation of hydroperoxides. Trommer et al. (2003) also reported that the different molecular weight size of HA showed nearly similar effects on the level of generated lipid peroxidation products in the thiobarbituric (TBA) assay.

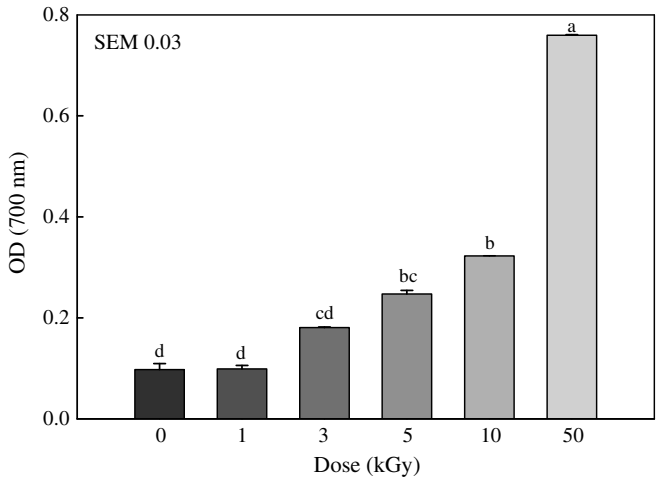

Fig. 4. Reducing power of gamma irradiated Hyaluronic acid (HA). Alphabet (a–d) are significantly different at $p \leqslant 0.05$. Standard error of the mean (SEM, $n = 12$).

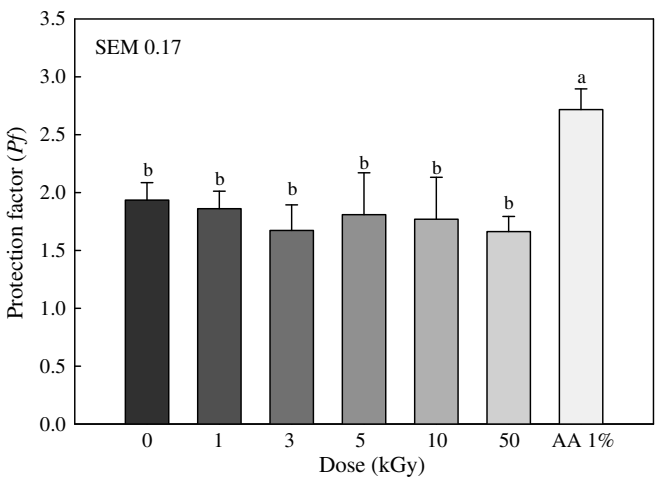

Fig. 5. Antioxidant effect (Pf) of gamma irradiated hyaluronic acid (HA) using Rancimat test. Alphabet (a, b) are significantly different at $p \leqslant 0.05$. Standard error of the mean (SEM, $n = 18$). Ascorbic acid (AA).

4. Conclusions

Several methods have been applied to depolymerise hyaluronic acid into lower molecular mass fragments and many studies have investigated the advantages of lower molecular weight of HA for certain applications in the fields such as medical treatments and cosmetics. In this study, gamma irradiation was used for depolymerisation and we studied the structural modification and antioxidant nature of irradiated HA.

Gamma irradiation decrease the molecular weight, viscosity, pH of hyaluronic acid and changed it to more intense yellowish colour. UV spectra of irradiated HA showed a new absorption band at 265 nm, which indicates the formation of double bonds. Differences in the height and shape of certain absorption bands of FT-IR spectra were observed in the spectral range at 1700–1750 cm^{-1}, which is associated with the formation of a carboxylic acid. These structural changes of gamma irradiated HA provide some evidence of the formation of a pyrancarboxylic acid ring. DPPH radical scavenging ability and reducing power of gamma irradiated HA were significantly higher than those of non-irradiated HA. However, non-irradiated and irradiated HA did not show significant differences in the Rancimat test. Therefore, gamma irradiation could be an alternative method for a depolymerisation with increasing antioxidant activity of HA.

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