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Antioxidant potential of Maillard reaction products formed by irradiation of chitosan–glucose solution

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ABSTRACT

Chitosan–glucose solution was exposed to gamma radiation to doses up to 100 kGy. Formation of Maillard reaction products (MRPs) was monitored by measuring changes in UV absorbance, browning and fluorescence. The amino group and reducing sugar content of the solution decreased with increasing dose of radiation suggesting reaction between carbonyl and amino groups. There was a marked increase in the reducing power of the solution which correlated with high DPPH and superoxide radical scavenging activity. Chitosan–glucose MRPs provided good protection to β-carotene against bleaching indicating its high antioxidant potential. 5-Hydroxymethylfurfural, a known cytotoxic product formed by heat-induced Maillard reaction was not detected in MRPs formed by irradiation of chitosan–glucose solution. These MRPs also showed antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas fluorescens* and *Bacillus cereus*.

1. Introduction

Chitosan is a deacetylated derivative of chitin extracted from exoskeletons of shrimp and shell fish. It is an abundant by-product of seafood industry. It is non-toxic, biocompatible and biodegradable. It exhibits antibacterial and antifungal activities and has therefore received attention as a potential food preservative of natural origin (Chen, Liau, & Tsai, 1998; Rao, Chander, & Sharma, 2005). Chitosan has also received attention as a promising biomass resource, and it has been studied extensively with regard to its industrial applications (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). As its chemical structure has an amino group, chitosan exhibits a polycationic nature. Several researchers have developed methods to improve the properties of chitosan using chemical and enzymatic modifications. However, chemical modifications are generally not preferred in food applications.

Maillard reaction is a very complex reaction between carbonyls and amines. It occurs spontaneously during food processing and storage. Maillard reaction usually produces a wide range of products with odors and colors, antioxidant, antiallergenic, antimicrobial and cytotoxic properties (Liu, Yang, Jin, Hsu, & Chen, 2008; Plavsic, Cosovic, & Lee, 2006; Rufian-Henares, Delgado-Andrade, & Morales, 2006). Apart from Maillard products generated in food materials during processing and storage, the Maillard reaction also occurs *in vivo* with important pathological consequences for biological systems (Thorpe & Baynes, 2003). Maillard reaction products (MRPs) of amino acid–sugar have been found to exhibit strong antioxidant activity. The MRPs produced from a casein–glucose model system by heat have been associated with the formation of compounds with strong antioxidant activity (Gu et al., 2009). Gamma irradiation is well established physical and non-thermal method for preservation and enhancement of microbiological safety of food. The safety and wholesomeness of irradiated foods has been well established and reviewed from time to time (Diehl, 2002). The majority of chemical changes caused due to radiation processing of food are similar to those by other preservation methods like heat (Diehl, 1995). Non-enzymatic browning in gamma-irradiated aqueous solutions of different sugars with lysine was reported by Oh et al. (2006). Ionizing radiation was reported to produce MRPs in aqueous glucose/amino acid solutions (Chawla, Chander, & Sharma, 2007) and antioxidant potential of MRPs formed by irradiation of whey proteins has been reported recently (Chawla, Chander, & Sharma, 2009). Chitosan has amino groups which can react with carbonyl group of reducing sugar leading to formation of MRPs. Monosaccharide derivatives of low molecular weight chitosan with glucosamine, N-acetyl galactosamine, galactose and mannose formed by Maillard reaction have been reported to exhibit antibacterial activity against *Escherichia coli* and *Bacillus subtilis* (Il'ina, Kulikov, Chalenko, Gerasimova, & Varlamov, 2008). The antibacterial activity of chitosan was revealed to be further enhanced by its Maillard reaction at 95 °C for 10 h and at pH 6 with xylose (Huang, Huang, Huang, & Chen, 2007). Chitosan–glucose MRPs prepared by heat treatment have been shown to improve the

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bacteriological quality of various meat products (Kanatt, Chander, & Sharma, 2008).

There is a growing scientific interest in the influence of irradiation processes on antioxidant activity and the compounds responsible for such activity. In the reaction, no other chemical reagents are introduced and there is no need to control temperature, environment or additives. Recently, radiation effects on carbohydrates such as chitosan, alginate, carrageenan, cellulose and pectin have been investigated to enhance their bioactivities and to reduce environmental pollution (Chmielewski, Haji-Saeid, & Ahmed, 2005). However, no information on irradiation of chitosan with glucose solution, formation of MRPs and resultant effects on antioxidant activity and antibacterial activity is available. The aim of this study was to use radiation as a tool to form MRPs in chitosan–glucose solution and study its antioxidant and antibacterial potential.

2. Materials and methods

2.1. Chemicals

Chitosan powder (degree of deacetylation of 78–82%, moisture content less than 10%) was provided by Mahatani Chitosan Pvt. Ltd. (Veraval, India). β-Carotene, 2,2,-diphenyl-1-picrylhydrazyl (DPPH), nitroblue tetrazolium, nicotinamide adenine dinucleotide reduced (NADH) and linoleic acid were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade and procured from Himedia Laboratories (Mumbai, India) or Sisco Research Laboratories (Mumbai, India).

2.2. Microorganisms

E. coli JM109, *Pseudomonas fluorescens* (lab isolate), *Staphylococcus aureus* ATCC 6538P, *Bacillus cereus* MTCC 470 cultures were maintained at 4 °C. The long-term storage of cultures was done in 20% glycerol (v/v) at 20 °C. The isolates were subcultured twice before inoculation.

2.3. Preparation of radiation induced MRPs

A 2% solution of chitosan was prepared in acetic acid (1%). Similarly, a 2% solution of glucose was prepared in distilled water. Both solutions were mixed to obtain chitosan–glucose (1%) solution. The chitosan–glucose solution was exposed to different doses of γ-irradiation (0–100 kGy) in a Gamma-cell 5000 (BRIT, Mumbai, India) supplying a dose rate of 7.87 kGy/h. Irradiated chitosan solution (1%) was also kept as control for spectrophotometric analyses. Dosimetry was performed by ceric-cerous dosimeter calibrated against Fricke dosimeter. Dosimetry intercomparison was carried out with National Standards established by Radiological Physics and Advisory Division (RP&AD), Bhabha Atomic Research Centre, Mumbai, India.

2.4. Spectrophotometric analyses

Spectrophotometric changes were monitored according to the method described by Chawla et al. (2009). The radiation treated chitosan–glucose and chitosan solutions were appropriately diluted and absorbance at 284 nm (early Maillard reaction products) and 420 nm (late Maillard reaction products) were measured using a UV–vis spectrophotometer. Fluorescence of samples was determined after 100-fold dilution. The fluorescence intensity was measured at an excitation wavelength of 365 nm and emission wavelength of 440 nm using a fluorescence spectrophotometer.

2.5. Determination of free amino group and reducing sugar content

Free amino group content was determined by ninhydrin method described by Doi, Shibata, and Matoba (1981). Glucosamine was used to prepare standard curve for amino group measurement. The reducing sugar measurement (glucose) was done by dinitrosalicyclic acid method (Miller, 1959). According to this method, the reducing sugar reduces dinitrosalicyclic acid and produces an orange color complex that can be quantified at 540 nm. A standard curve plotting glucose concentration versus absorbance allows determination of concentration of reducing sugar in the system.

2.6. Determination of reducing power

Reducing power was determined by ferricyanide method described by Yen and Duh (1993). Appropriately diluted sample (1 ml) was added to 2.5 ml of phosphate buffer (200 mM, pH 6.6) followed by 2.5 ml of 1% potassium ferricyanide. The reaction mixture was incubated for 20 min in a water bath at 50 °C. After incubation, 2.5 ml of 10% trichloroacetic acid was added, followed by centrifugation at 3000 rpm for 10 min. The upper layer (5 ml) was mixed with 5 ml distilled water and 1 ml of 0.1% ferric chloride. Absorbance of the resultant solution was measured at 700 nm. A high absorbance was indicative of strong reducing power.

2.7. Determination of radical scavenging activity

Electron-donating ability of radiation induced MRPs was determined by employing DPPH radical scavenging assay (Kanatt et al., 2008). To 1 ml aliquot of appropriately diluted solution, 1 ml of ethanolic DPPH solution (0.2 mM) was added. The mixture was vortexed and left to stand at ambient temperature for 20 min. Reaction mixture containing 1 ml distilled water and 1 ml of ethanolic DPPH solution served as control. The absorbance of the solution was measured at 517 nm.

Superoxide anion scavenging activities of radiation induced MRPs were determined according to method described by Chawla et al. (2007) with some modifications. The reaction mixture consisted of 1 ml of nitroblue tetrazolium (156 μM in 0.1 M potassium phosphate buffer, pH 7.4), 1.0 ml of NADH (468 μM in 0.1 M potassium phosphate buffer, pH 7.4) and 0.5 ml of appropriately diluted sample. The reaction was initiated by addition of 100 μl of phenazine methosulphate (60 μM in 0.1 M potassium phosphate buffer, pH 7.4) to the mixture. The tubes were incubated at ambient temperature for 5 min and the absorbance was measured at 560 nm. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

The radical scavenging activity of DPPH and superoxide anion was calculated using the following formula:

$$\text{radical scavenging activity } \% = \left[\frac{A_o - A_s}{A_o}\right] \times 100$$

where A_o is absorbance of the control and A_s is absorbance of the sample.

2.8. Determination of antioxidant activity by β-carotene bleaching assay

Antioxidant activity of the aqueous solution was determined by a β-carotene/linoleic acid system as described by Matthaus (2002). Briefly, 1 ml of β-carotene solution (1 mg/ml in chloroform), 40 μl of linoleic acid, and 400 μl of Tween 80 were transferred into a round bottom flask. Chloroform from the sample was evaporated using a stream of nitrogen. Then, 100 ml of distilled water was

added slowly to the residue and vigorously agitated to give a stable emulsion. To an aliquot of 4.5 ml of this emulsion, 500 μl of appropriately diluted samples were added. To the control reaction mixtures, 500 μl of distilled water was added. Absorbance was measured immediately at 470 nm. The tubes were placed in a water bath at 50 °C and the absorbance was measured after 120 min. Antioxidant activity index (AAI) was calculated as:

$$\mathrm{AAI} = \frac{A_{s(0)} - A_{s(120)}}{A_{b(0)} - A_{b(120)}} \times 100$$

where $A_{s(0)}$ and $A_{b(0)}$ are absorbance of sample and blank at 0 min respectively. $A_{s(120)}$ and $A_{b(120)}$ are absorbance of sample and blank at 120 min respectively.

2.9. Detection of 5-hydroxymethylfurfural

5-Hydroxymethylfurfural was detected by using reverse phase HPLC with UV detection at 284 nm, according to the method proposed by Chávez-Servín, Castelotte, and López-sabater (2005). The HPLC system consisted of Quarternary Gradient Pump of Jasco PU-2089 plus. Separation was performed on ODS-hypersil C_{18} column (250 mm × 4.6 mm), with a 5 μm particle size (Thermo Electron Corporation, USA). The samples were filtered and separations were carried out isocratically at room temperature, using a mixture of acetonitrile–water (5:95, v/v) at a flow-rate of 1 ml/min for the mobile phase. The injection volume was 20 μl and the run time was 10 min. 5-Hydroxymethylfurfural (20 μg/ml) was used as standard and MRPs formed by heating of chitosan–glucose solution were used as positive control.

2.10. Antibacterial activity

Various food spoilage and pathogenic bacteria were used to evaluate the antibacterial activity of chitosan–glucose Maillard reaction products (CGMRPs) formed by 100 kGy. This particular sample was chosen to evaluate antibacterial activity as it showed maximum antioxidant activity. Unirradiated chitosan solution was used as positive control. Nutrient broth was inoculated with the test culture and incubated overnight at 37 °C. Different volumes of CGMRPs and chitosan were added to the phosphate buffered saline (PBS, pH 7.2) tubes in order to get a final concentration of 0.01%. The cells were washed twice with phosphate buffered saline (PBS) (pH 7.2) and re-suspended in buffer containing antibacterial solutions. A tube containing only the test culture served as the control. At the initial point (0 h) a sample was withdrawn, serial dilutions carried out, plated on plate count agar (by spread plate method) and counted after incubation at 37 °C for 18 h. This gave the initial number of the test organism (expressed as log cfu/ml). All the tubes were then incubated for 24 h at 37 °C, the aliquots were again taken and the surviving population was determined. The antibacterial activity of CGMRPs and chitosan was assessed by decrease in log cfu/ml of the test culture in 24 h.

2.11. Statistical analyses

All results given in the figures are mean and standard deviation of three experiments. Differences between the variables were tested for significance by one-way ANOVA with Tukey's post-test using GraphPad InStat version 3.05 for window 95, GraphPad Software, San Diego, CA, USA, www.graphpad.com. Differences at $P < 0.05$ were considered to be significant.

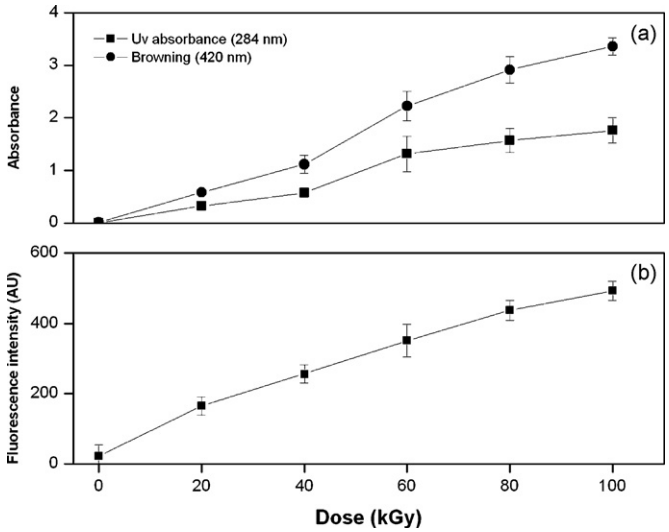

Fig. 1. (a) UV absorbance (284 nm), browning (420 nm) and (b) fluorescence of irradiated chitosan–glucose solution at various irradiation doses. The results shown are mean ± SD of three independent experiments.

3. Results and discussion

3.1. Formation of chitosan–glucose MRPs

An increase in UV absorbance of chitosan–glucose solution was observed with increasing radiation dose (Fig. 1a). Maillard reaction is associated with development of UV-absorbing intermediate compounds, prior to generation of brown pigments. UV-absorbing intermediate compounds are formed prior to radiation induced MRPs (Chawla et al., 2007). The result suggested that intermediate products were produced to a great extent. In the final stage, the intermediates polymerize and colored polymers are formed. It can be seen that the browning intensity for chitosan–glucose solution increased with the radiation dose (Fig. 1a). This suggested that irradiation may lead to non-enzymatic browning reactions, similar to those induced by heating (Nicoli, Casadei, Guerzoni, & Lerici, 1994). Browning was also observed in irradiated chitosan solution but, there was no significant increase in UV absorbance (results not shown). The Maillard reaction is also associated with the development of fluorescent compounds. Dose-dependent formation of fluorescent compounds was observed in irradiated chitosan–glucose solution suggesting formation of CGMRPs (Fig. 1b). Similar radiation dose-dependent increase in UV absorbance, browning and florescence has been reported in sugar–amino acid solution and whey protein dispersion (Chawla et al., 2007, 2009).

3.2. Reduction in amino group and reducing sugar

Changes in reactive amino groups of MRPs after irradiation are depicted in Fig. 2a. Initially, few reactive amino groups were detected in chitosan–glucose solution as chitosan is a polymer with extensive hydrogen bonding. Upon irradiation the hydrogen bonds are broken and amino groups are exposed. A continuous decrease in amino group content of all MRP samples was noticed as the radiation dose increased ($P < 0.05$). This result suggested that NH_2 group of chitosan covalently attached to glucose to form glycated product to a greater extent, particularly when the dose is increased. From the results, it is obvious that the decrease in free amino group was in accordance with the increase in browning and UV absorbance at 284 nm (Fig. 1a). This indicated that radiation catalyzed the interaction between free amino groups of chitosan and

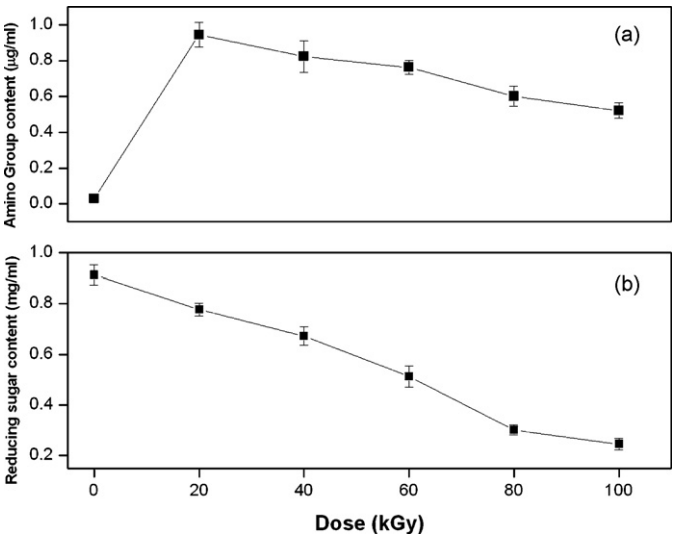

Fig. 2. Reduction in amino group content (a) and reducing sugar content (b) of chitosan–glucose solution subjected to different doses of gamma radiation. The results shown are mean ± SD of three independent experiments.

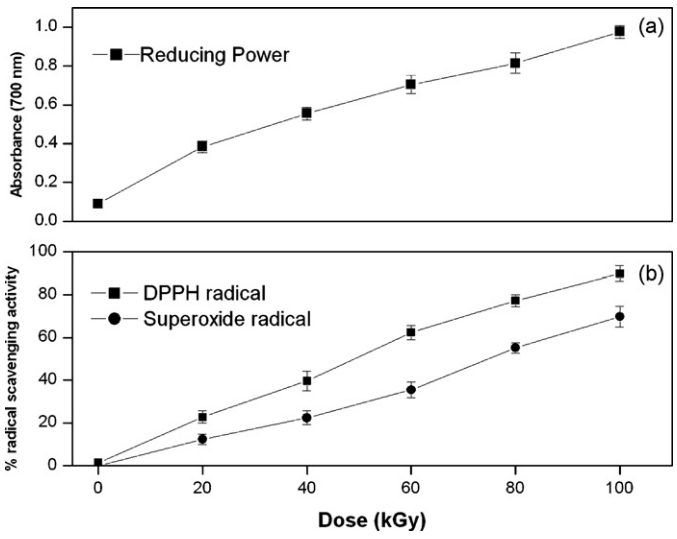

Fig. 3. Reducing power (a) and radical scavenging activity (b) of CGMRPs as function of irradiation dose. The results shown are mean ± SD of three independent experiments.

glucose via glycation process. As a result, intermediate products were formed and further converted to brown pigments, as observed by the increased absorbance at 420 nm. The changes in reducing sugar content of chitosan–glucose solution, as a function of irradiation dose, are shown in Fig. 2b. A significant dose-dependent decrease in the reducing sugar content was observed in irradiated chitosan–glucose solution. Reduction in reducing sugar content during heat-induced Maillard reaction in fructose/lysine and radiation induced Maillard reaction in whey protein dispersion (Chawla et al., 2009) has been reported. These results indicated the involvement of amino group and carbonyl group of sugar in formation of CGMRPs during irradiation treatment, as substantiated by the lower free amino groups and reducing sugars remaining upon irradiation treatment. It is known that the Maillard reaction is mainly divided into three stages (Martins, Van Jongen, & Boekel, 2001). Initial reaction between aldose sugar and an amino compound leads to the formation of the Amadori product via the Schiff base. In the subsequent reaction, the Amadori product is broken down into numerous products. In the final stage, the reaction products condense with amino compounds and the brown nitrogenous polymers and copolymers are finally formed.

3.3. Reducing power of chitosan–glucose MRPs

Reducing power of CGMRPs formed due to irradiation is shown in Fig. 3a. It was seen that unirradiated chitosan–glucose solution had negligible reducing power, whereas, upon irradiation, the reducing capacity of solution increased significantly. Reducing power of CGMRPs increased with the dose of irradiation, as shown by an increase in absorbance at 700 nm ($P < 0.05$). It has been reported that compounds responsible for reducing activity are formed during thermolysis of Amadori products in the primary phase of Maillard reaction (Hwang, Shue, & Chang, 2001) or could be heterocyclic products of Maillard reaction (Charurin, Ames, & Castiello, 2002). Additionally, the intermediate reductone compounds of MRPs were reported to break the radical chain by donation of a hydrogen atom (Eichner, 1981). Possibly, gamma-irradiation induces similar changes in chitosan–glucose solution, resulting in the formation of products which contribute towards the reducing power. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.

3.4. Radical scavenging activity

DPPH is a chromogen-radical containing compound that can directly react with antioxidants. When the DPPH radical is scavenged by antioxidants through the donation of hydrogen to form a stable DPPH-H molecule, the color is changed from purple to yellow. Stable radical DPPH has been widely used for the determination of primary antioxidant activity, that is, the free radical scavenging activities of pure antioxidant compounds, plant and fruit extracts, and food materials (Shih, Lai, & Jen, 2006). The changes in the DPPH radical scavenging activity of MRPs derived from chitosan–glucose solution, as a function of irradiation dose are shown in Fig. 3b. The DPPH radical scavenging activity of CGMRP samples was increased with an increase in radiation dose. CGMRPs formed at 100 kGy dose have 89% DPPH radical scavenging activity. DPPH radical scavenging activity correlated well with the browning intensity and absorbance at 284 nm. These results are in agreement with those of Yen and Hsieh (1995), who showed that MRPs possessed DPPH radical scavenging activity.

CGMRPs formed by irradiation effectively scavenged superoxide radical. Linear increase in superoxide radical scavenging was observed with increasing radiation dose. CGMRPs obtained by 100 kGy dose scavenged up to 70% of superoxide radicals in the system (Fig. 3b). The superoxide radicals are generated by a number of biological reactions. Although they do not directly initiate lipid oxidation, superoxide radical anions are precursors of highly reactive hydroxyl radical, which contributes towards lipid peroxidation in biological systems. Thus, superoxide anion scavenging activity indirectly contributes towards antioxidant potential. Formation of compounds that are capable of scavenging hydroxyl and superoxide anion radicals, as result of radiation induced MR in sugar/amino acid model systems, has been reported (Chawla et al., 2007).

3.5. β-Carotene bleaching activity

It was observed that β-carotene bleaching was significantly ($P < 0.05$) inhibited in the presence of irradiated chitosan–glucose solution, whereas, no protection was offered by non-irradiated solution (Fig. 4). The mechanism of bleaching of β-carotene is a free radical-mediated phenomenon, resulting from the hydroperoxides formed from linoleic acid. In this model system, β-carotene undergoes rapid discoloration due to attack of free radicals formed

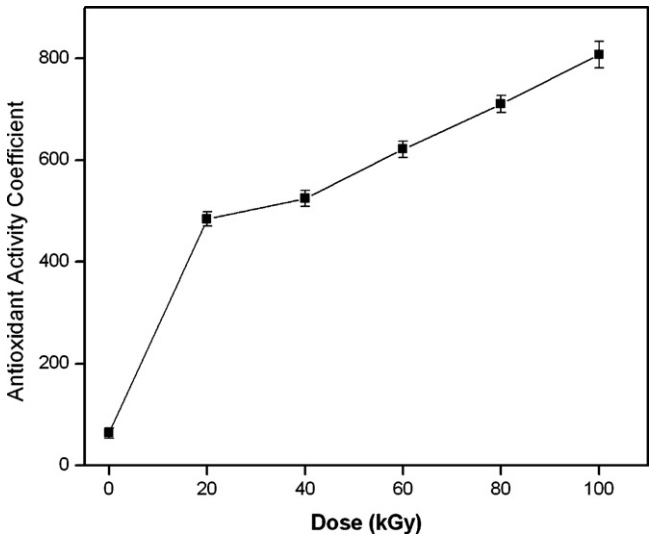

Fig. 4. Antioxidant activity of MRPs formed by radiation treatment of chitosan–glucose solution as assessed by β-carotene bleaching assay. The results shown are mean ± SD of three independent experiments.

upon abstraction of a hydrogen atom from the diallylic methylene group of linoleic acid. The presence of an antioxidant in the reaction mixture hinders the rate of bleaching by neutralizing free radicals formed in the system during incubation at 50 °C. Synthesis of antioxidant compounds upon heat treatment of sugar/amino acid solutions has been reported (Lingnert and Eriksson, 1980). Thus, these findings indicated that compounds formed upon irradiation of chitosan–glucose solution have significant antioxidant potential.

3.6. 5-Hydroxymethylfurfural detection

A peak corresponding to 5-hydroxymethylfurfural was obtained at 8.2 min in case of heat-induced Maillard reaction, whereas, no 5-hydroxymethylfurfural was detected in CGMRPs formed by 100 kGy dose (Fig. 5). 5-Hydroxymethylfurfural was not detected in any of the CGMRPs formed by irradiation at lower doses (results not shown). 5-Hydroxymethylfurfural is intermediate product of the Maillard reaction (Ferrer, Algría, Farré, Abellán, & Romero, 2002). 5-Hydroxymethylfurfural at high concentrations is cytotoxic, causing irritation to eyes, upper respiratory tract, skin and mucous membranes; an oral LD_{50} of 3.1 g/kg body weight has been determined in rats (Ulbritch, Northup, & Thomas, 1984). Oh et al. (2006) reported that no furfurals were detected in irradiated sugar–amino acid solution, whereas, these compounds were found in heated sugar–amino acid solutions. These results corroborate their findings that irradiation of sugar–amino acid solution does not lead to formation of 5-hydroxymethylfurfural.

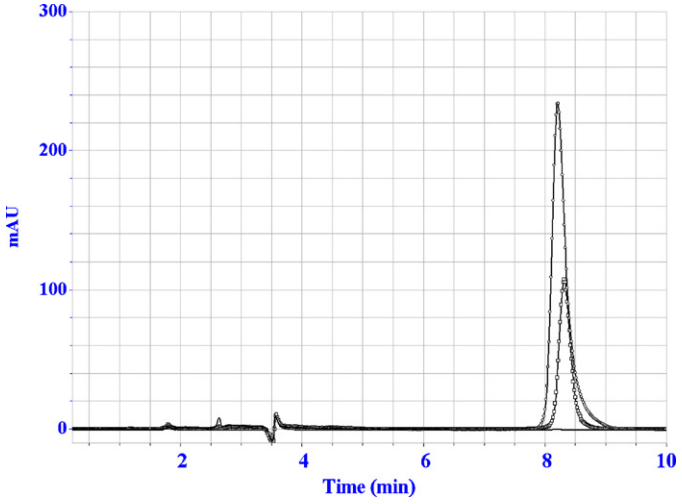

Fig. 5. HPLC profile of HMF (□, MRPs formed by heat treatment (○) and CGMRPs formed by irradiation (—).

Table 1
Antibacterial activity of CGMRPs and chitosan.

Organism	Initial count (0 h)	Sample[a]		
		Control	CGMRPs[b]	Chitosan[b]
E. coli	7.82 ± 0.25	7.56 ± 0.28	2.42 ± 0.10	2.98 ± 0.21
P. fluorescens	8.45 ± 0.34	8.20 ± 0.32	4.36 ± 0.38	5.01 ± 0.41
S. aureus	7.45 ± 0.34	7.23 ± 0.39	3.86 ± 0.37	4.15 ± 0.21
B. cereus	6.23 ± 0.56	6.00 ± 0.45	4.14 ± 0.51	4.78 ± 0.29

[a] The values reported are log cfu/ml after 24 h.
[b] The concentration of CGMRPs and chitosan was 0.01% (v/v).

3.7. Antibacterial activity

The antibacterial activity of CGMRPs (100 kGy) and chitosan is shown in Table 1. MRPs formed by irradiation of chitosan–glucose solution were effective against all the bacterial cultures used in this study, however the extent of inhibition varied. Maximum antibacterial activity was seen against *E. coli* where five log cycle reduction was observed. In case of *S. aureus* and *P. fluorescens*, CGMRPs led to three log cycle decrease in colony forming units. Lowest activity was seen against *B. cereus* as only one log cycle reduction was seen. The mechanism of growth inhibition by chitosan is not fully understood, but is generally attributed to its polycationic nature which disrupts the membrane integrity. Chung, Kuo, and Chen (2005) have reported relatively high antibacterial activity against *E. coli* and *S. aureus* for the chitosan–glucosamine derivatives, produced using the Maillard reaction as compared with native chitosan. Soy protein–chitosan conjugate, formed by the Maillard reaction, has also been reported to enhance bactericidal action (Usui et al., 2004). We observed statistically insignificant decrease in antibacterial activity of CGMRPs as compared to native chitosan. The decrease in antibacterial activity of chitoligosaccharides formed by irradiation of chitosan solution due to degradation of polymer has been reported earlier (Rao, Chander, & Sharma, 2008).

4. Conclusion

Ionizing radiations, such as gamma rays, can be successfully employed to produce MRPs in chitosan–glucose solution. The results of the present study demonstrated the formation of CGMRPs with antioxidant potential when chitosan–glucose solution was irradiated. To the best of our knowledge it is the first report where we have shown the formation of MRPs having potential antioxidant activity by irradiation of chitosan–glucose solution without formation of 5-hydroxymethylfurfural which is known to be cytotoxic. Along with antioxidant property, radiation induced CGMRPs also showed antibacterial activity against various bacteria. This study demonstrated the safety of irradiation as a method to form MRPs with potential antioxidant and antibacterial properties in food systems. Further studies are needed to elucidate the mechanism of compounds formed during radiation induced Maillard reaction and their identification. Also, efforts are needed to investigate the factors affecting the reaction and potential applications of such MRPs in food systems as preservatives.

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