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Chitosan glucose complex – A novel food preservative

Sweetie R. Kanatt, Ramesh Chander *, Arun Sharma

Food Technology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085, India

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Abstract

Chitosan glucose complex (CGC), a modified form of chitosan was prepared by heating chitosan with glucose. Fluorescence and the browning reaction of CGC indicated the presence of the Maillard reaction product (MRP). CGC showed excellent antioxidant activity while chitosan or glucose alone did not have any significant activity ($p < 0.05$). The IC_{50} value of CGC for DPPH radical scavenging was 51.1 μg/ml. The efficacy of CGC in scavenging hydroxyl and superoxide anion radicals was also very high but it showed a low reducing power. The antimicrobial activity of CGC was similar to chitosan against *E. coli*, *Pseudomonas*, *Staphylococcus aureus* and *Bacillus cereus*, the common food spoilage and pathogenic bacteria. The minimum inhibitory concentration of CGC or chitosan was 0.05%. Addition of CGC to lamb meat increased its shelf life by more than 2 weeks during chilled storage. It also enhanced the shelf life of pork cocktail salami to 28 days. CGC is endowed with both antioxidant and antimicrobial activity and thus is a promising novel preservative for various food formulations.

Keywords: Chitosan glucose complex; Maillard reaction; Antioxidant; Antimicrobial

1. Introduction

Chitosan (deacetylated chitin), a polycationic biopolymer is commercially prepared from shellfish-processing waste and is non-toxic, biodegradable and biocompatible (Rudrapatnam & Farooqahmed, 2003). It exhibits antibacterial and antifungal activity and has therefore received attention as a potential food preservative of natural origin (Chen, Liau, & Tsai, 1998; El Ghaonth, Arul, Asselin, & Benhamon, 1992; Rao, Chander, & Sharma, 2005; Roller & Covill, 2000). Studies in our laboratory have shown that chitosan exhibits excellent antibacterial activity but is ineffective in preventing oxidative rancidity (Kanatt, Chander, & Sharma, 2004; Rao et al., 2005).

Several researchers have developed methods to improve the properties of chitosan using chemical and enzymatic modifications. However, chemical modifications are generally not preferred for food applications because of the formation of potential detrimental products. Chitosan–lysozyme conjugates have been reported to have better emulsifying properties and bactericidal action (Song, Babiker, Usui, Saito, & Kato, 2002).

The Maillard reaction, resulting from condensation between the carbonyl group of reducing sugars, aldehydes or ketones and an amine group of amino acids, proteins or any nitrogenous compound, is one of the main reactions taking place in food (Hodge & Rist, 1953). Maillard reaction compounds contribute to flavour formation, antioxidative and antimicrobial effects and improvement of functional properties (Chevalier, Chobert, Genot, & Haertle, 2001; Chuyen, 1998). It is desirable to modify chitosan so that it attains excellent antioxidant activity without affecting its antimicrobial activity. Chitosan has an amino group which can react with the carbonyl group of a reducing sugar. Hence, chitosan was heated with glucose to form a Maillard reaction product. The aim of the present study was to investigate the antioxidant and antibacterial property of this chitosan–glucose complex (CGC) and to

* Corresponding author. Tel.: +91 22 25593296/25595374; fax: +91 22 25505151/25519613.
E-mail address: rchander@barc.gov.in (R. Chander).

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investigate its efficiency as a preservative in meat and meat products.

2. Materials and methods

2.1. Chemicals

Chitosan was obtained, in powder form, from Mahatani Chitosan Pvt. Ltd. (Veraval, India). It had a deacetylation range of 78–82% and the moisture content was less than 10% (manufacturer's data). Butylated hydroxy toluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Nitroblue tetrazolium (NBT) were purchased from Sigma Chemical Co. (St. Louis, MO). Phenazine methosulphate (PMS), deoxyribose and nicotinamide adenine dinucleotide (NADH) disodium salt were purchased from HiMedia (India). All other reagents used were of analytical grade and procured from Qualigens Fine Chemicals (Mumbai, India) and Sisco Research Lab (Mumbai, India).

2.2. Bacterial cultures

E. coli JM109, *Pseudomonas* spp. (lab isolate), *Staphylococcus aureus* ATCC 6538P and *Bacillus cereus* MTCC 470 cultures were stored in 20% glycerol (v/v) at −20 °C. Before the start of the experiment the cultures were grown on nutrient agar. The isolates were subcultured twice before inoculation.

2.3. Preparation of chitosan glucose complex

1% chitosan was prepared in 1% glacial acetic acid. CGC was prepared by autoclaving chitosan (1%) and glucose (1%) for 15 min.

2.4. Spectrophotometric analysis

The UV absorbance and browning of the sample was measured according to (Ajandouz, Tchiakpe, Ore, Benajiba, & Puigserver, 2001). Appropriate dilutions were made with distilled water and the absorbance was measured at 294 nm and 420 nm, for determining UV absorbance and browning intensity, respectively.

Suitable dilutions of the sample were taken for measurement of fluorescence as described by Morales and Jimenez-Perez (2001). The fluorescent intensity was measured at an excitation wavelength of 347 nm and an emission wavelength of 415 nm using a Jasco FP-6500 fluorescence spectrophotometer.

2.5. Scavenging of free radicals

The radical scavenging activity was determined by using the stable DPPH radical (Yamaguchi, Takamura, Matoba, & Terao, 1998). The diluted sample (200 μl) was mixed with 800 μl of Tris-HCl buffer (100 mM, pH 7.4). To this was added 1 ml of 500 μM DPPH in ethanol (final concentration of 250 μM) and the mixture was vortexed vigorously. After 20 min, incubation in the dark, the absorbance was measured at 517 nm. Percent DPPH scavenging activity was calculated as

$$[(\text{Control absorbance} - \text{Extract absorbance})/(\text{Control absorbance})] \times 100.$$

2.6. Scavenging of hydroxyl radical

Non-site specific hydroxyl radical scavenging activity was determined according to the deoxyribose method of (Halliwell, Gutteridge, & Aruoma, 1987), in the presence of EDTA. Briefly, to 1 ml of appropriately diluted sample, 1 ml phosphate buffer (0.1 M pH 7.4) containing 1mM ferric chloride, 1 mM EDTA, 1 mM ascorbic acid, 30 mM deoxyribose and 20 mM hydrogen peroxide, were added. Following incubation at 37 °C for 90 min, 2 ml of 2% (w/v) TCA and 2 ml of 1% (w/v) TBA were added to the reaction mixture which was then heated in a boiling water bath for 15 min. The absorbance of the pink colour which developed was measured at 532 nm, using a spectrophotometer. Site-specific hydroxyl radical scavenging activity of sample was performed as described above except that EDTA was absent in the reaction system. The percent inhibition of hydroxyl radical was calculated as above for the DPPH assay.

2.7. Super oxide anion radical scavenging activity

The generation of super oxide anion radicals was carried out as described by (Liu, Ooi, & Chang, 1997), with some modifications. The reaction mixture consisted of 1 ml of NBT (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 an appropriately diluted sample. To initiate the reaction 100 μl of PMS (60 μM in 0.1 M potassium phosphate buffer pH 7.4) was added to the mixture. The absorbance was measured at 560 nm, after incubation at ambient temperature for 5 min. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula

$$\%\ \text{Inhibition} = [(A_o - A_s)/A_o] \times 100$$

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

2.8. Measurement of reducing power

The reducing power of the samples was determined according to the method of Oyaizu (1986). An aliquot of the sample (2.5 ml) was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potas-

sium ferricyanide and the mixture was incubated at 50 °C for 20 min. Ten percent TCA (2.5 ml) was added and the mixture was centrifuged at 650 g for 10 min. The supernatant (5 ml) was mixed with 5 ml of D/W and 1 ml of 0.1% ferric chloride and the absorbance was measured at 700 nm.

2.9. Assay for antibacterial activity

The activity of chitosan and CGC was measured against a number of food spoilage and pathogenic bacteria. Nutrient broth (25 ml) was inoculated with the test culture and incubated overnight at 37 °C. Different volumes of CGC and chitosan were added to the nutrient broth tubes in order to get a final concentration of 0.01%, 0.05%, 0.1% and 0.2%. The test culture (0.1%) was then inoculated into the nutrient broth tubes containing CGC/chitosan. 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 4 h and 24 h at 37 °C, the aliquots were again taken and the surviving population was determined. The antibacterial activity of CGC/chitosan was assessed by decrease in log cfu/ml of the test culture in 24 h.

2.10. Effect of CGC/chitosan on chilled storage of minced lamb meat and pork cocktail salami

A leg portion of lamb meat was taken, all the visible fat removed, minced in a food processor (Sumeet, India) and then divided into three batches. Chitosan (0.1%) was added to the first batch, mixed thoroughly and then packed in polythene pouches. To the second batch CGC (0.1%) was added while the third batch served as the control. All the packets were then stored at 0–3 °C. The total viable counts of the control and treated samples were determined immediately and at intervals of one week. CGC (0.1%) was also incorporated in the meat formulation for the commercial preparation of pork cocktail salami in Hygienic Meat Products, Mumbai. The salamis were then stored at 0–3 °C and the total counts determined weekly.

2.11. Statistical analysis

All experiments were carried out in triplicate and average values with standard errors are reported. Analysis of variance was conducted and differences between variables were tested for significance, by one-way ANOVA, with Tukey's post test using GraphPad InStat version 3.05 for Windows 95, GraphPad Software, San Diego California USA, <www.graphpad.com>. A statistical difference at $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Fluorescence and browning

Fluorescent products have been used to measure the level of MRPs formed. The fluorescence intensity of the CGC was found to be high. At a concentration of 0.1% CGC, fluorescence intensity was 896. Fluorophores formed during the Maillard reaction are the precursors of the brown pigments (Adhikari, 1973; Jing & Kitts, 2002). During the formation of CGC on heating, there was a visual colour change, from uncoloured to dark brown. The reaction temperature of 121 °C, in the present study, was selected to represent the sterilisation temperature. Increase in browning as observed by absorbance at 420 nm was seen in the CGC (Fig. 1). It could be observed that CGC also gave high UV absorbance at 294 nm (Fig. 1) and this is indicative of the formation of intermediate compounds of the Maillard reaction (Ajandouz et al., 2001). Therefore, the fluorescence and browning reaction of CGC indicated the formation of MRP.

3.2. DPPH radical scavenging activity

The relatively stable DPPH radical has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and thus to evaluate the antioxidant activity (Jao & Ko, 2002). Fig. 2 shows the percentage DPPH free radical scavenging activity of CGC/chitosan/glucose. CGC is endowed with very good antioxidant activity ($p < 0.05$), while glucose or chitosan alone did not have any scavenging potential. The antioxidant efficiency of CGC was dependent on its concentration. The IC_{50} value of CGC, which is the concentration at which 50% scavenging of the free radical is obtained, was found to be 51.1 μg/ml. Therefore, CGC possesses hydrogen donating ability, suggesting potency to react with free radicals. Products of browning reactions have been shown

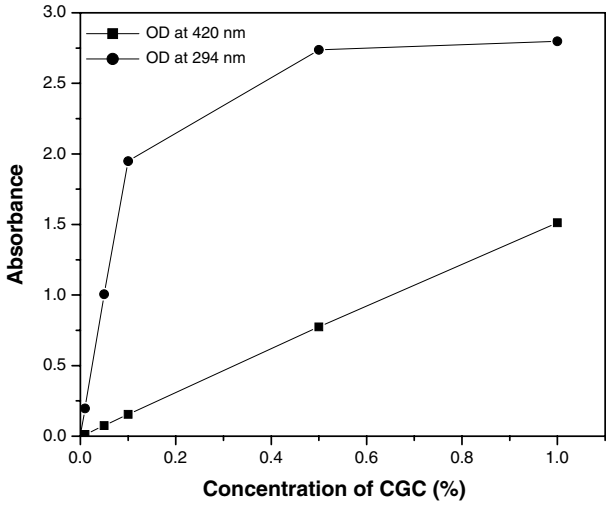

Fig. 1. Browning reaction and UV absorption of CGC.

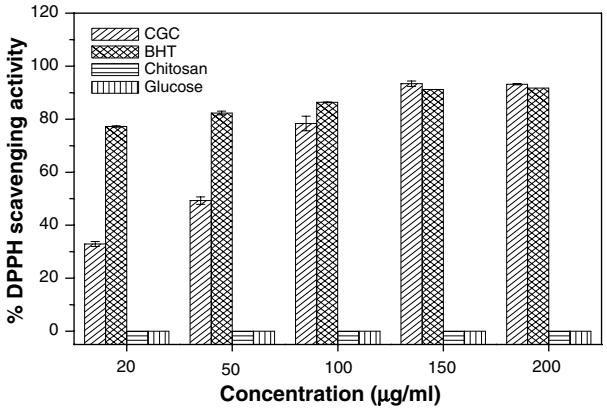

Fig. 2. DPPH radical scavenging activity of CGC. Values are the mean ± standard deviation of three replicate experiments.

to possess antioxidant activity (Benjakul, Visessanguan, Phongkanpai, & Tanaka, 2005; Morales & Jimenez-Perez, 2001; Yoshimura, Iijima, Watanabe, & Nakazawa, 1997).

3.3. Hydroxyl and Superoxide radical scavenging activity

The effect of CGC and chitosan/glucose on deoxyribose attack by a $OH^{\cdot}$ radical is depicted in Fig. 3. CGC could quench the hydroxyl radicals generated in non-site specific assay effectively, while chitosan and glucose did not show any activity (Fig. 3a). There was a linear correlation ($r^2 = 0.99$, $p < 0.001$) between concentration of CGC and $OH^{\cdot}$-scavenging activity. The effectiveness of CGC in inhibiting deoxyribose degradation was less in the site-specific assay (Fig. 3b). According to (Xie, Xu, & Liu, 2001), chitosan derivatives could scavenge hydroxyl radicals via hydrogen transmission mechanisms in which the OH or NH_2 groups in the pyranose ring of chitosan were implied as the centers of radical scavenging behaviour. MRPs have been reported to have antioxidant activity through scavenging oxygen radicals (Wijewickreme, Krejpcio, & Kitts, 1999; Yoshimura et al., 1997).

The superoxide radical ($O_2^{\cdot-}$), a potential precursor of highly reactive species such as the hydroxyl radical is generated in various biological reactions. Superoxide radicals were generated in a PMS-NADH system and assayed by the reduction of NBT. Fig. 4 illustrates the superoxide radical scavenging ability of CGC. There was a concentration dependent scavenging of superoxide radicals and CGC had an IC_{50} value of 236.13 μg/ml. Chitosan or glucose alone did not show any significant ($p < 0.05$) scavenging of superoxide radicals.

3.4. Reducing power

Different studies have indicated that the antioxidant effect is related to the development of reductones, which are terminators of free radical chain reactions (Singh & Rajini, 2004; Dorman, Kosar, Kahlos, Holm, & Hiltunen, 2003). The reducing activity of CGC is shown in Fig. 5. CGC did not show significant reducing power. Even at a concentration of 3.75 mg/ml CGC did not show any noteworthy reducing activity. Benjakul et al. (2005) reported that MRPs derived from a porcine plasma protein–sugar model system, prepared with glucose, exhibited the lowest reducing power, while those with galactose showed the highest reducing power.

3.5. Antimicrobial activity

The inhibition of test organisms by CGC/chitosan is depicted in Figs. 6–9. Addition of CGC/chitosan dramatically reduced the number of the following test organisms.

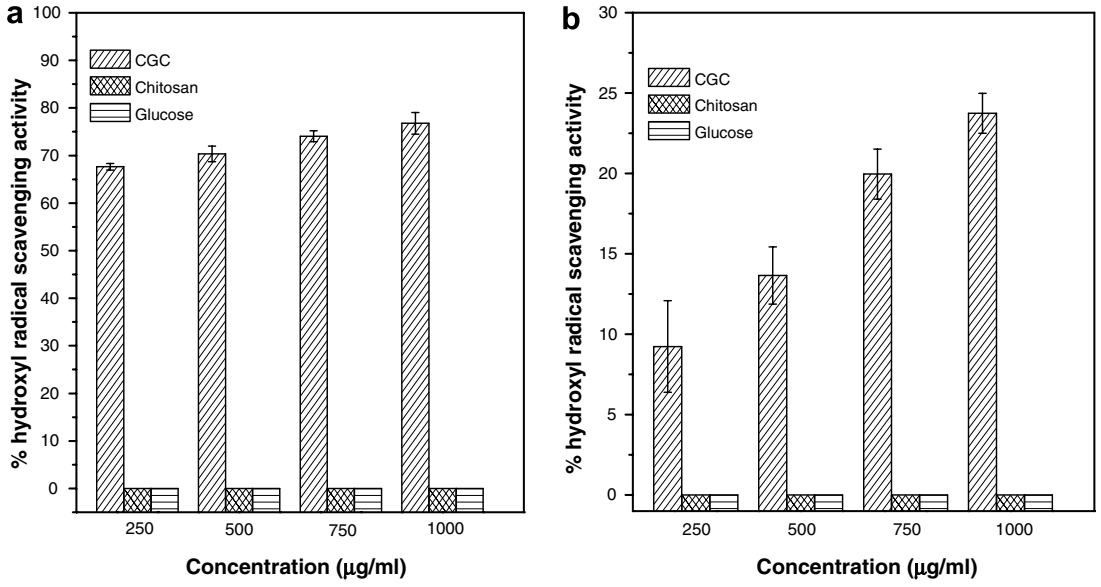

Fig. 3. Non-site specific (a) and site-specific (b) hydroxyl scavenging activity of CGC. Values are the mean ± standard deviation of three replicate experiments.

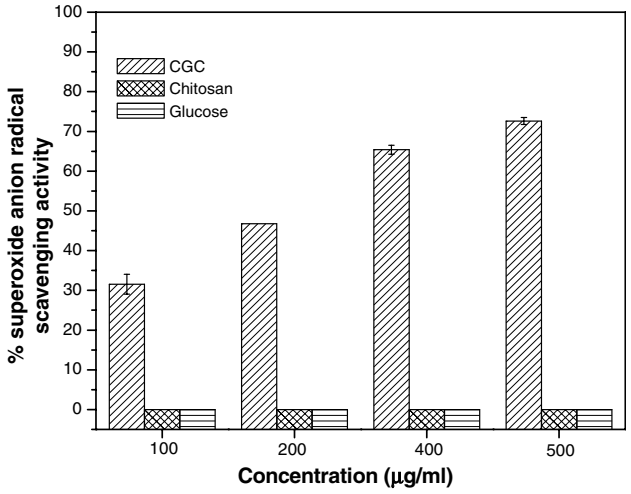

Fig. 4. Superoxide anion radical scavenging activity of CGC. Values are the mean ± standard deviation of three replicate experiments.

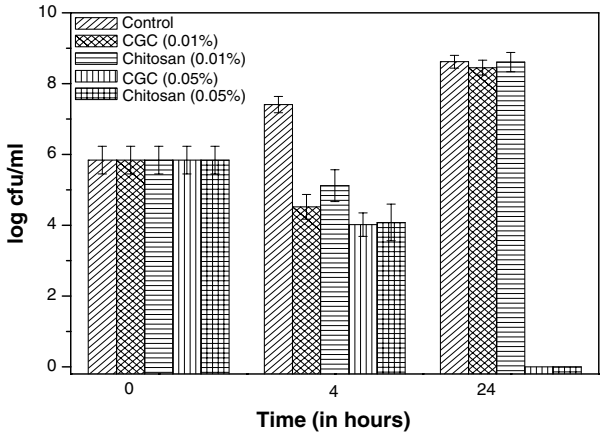

Fig. 7. Antimicrobial activity of CGC/chitosan against *Pseudomonas* measured in the nutrient broth. At concentrations above 0.01% CGC/chitosan completely inhibited the growth of the test culture. Results are mean values of three independent experiments.

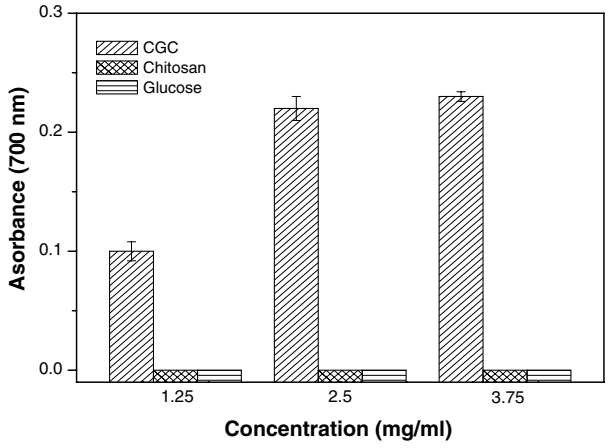

Fig. 5. Reducing power of CGC. Values are the mean ± standard deviation of three replicate experiments.

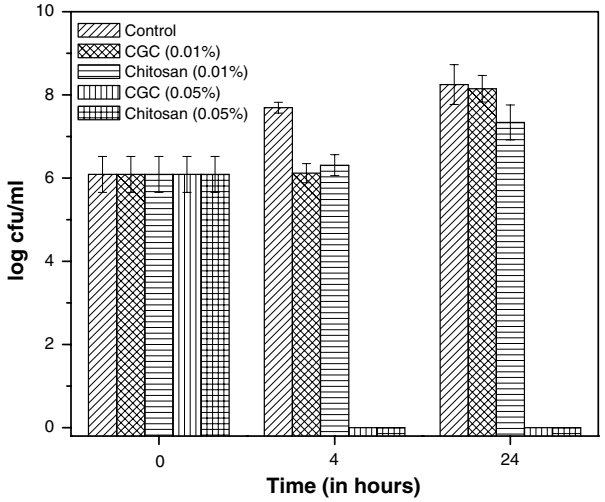

Fig. 8. Antimicrobial activity of CGC/chitosan against *S.aureus* measured in the nutrient broth. At concentrations above 0.01% CGC/chitosan completely inhibited the growth of the test culture. Results are mean values of three independent experiments.

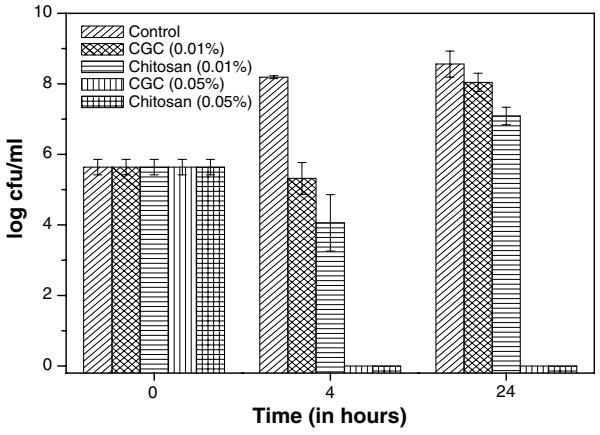

Fig. 6. Antimicrobial activity of CGC/chitosan against *E. coli* measured in the nutrient broth. At concentrations above 0.01% CGC/chitosan completely inhibited the growth of the test culture. Results are mean values of three independent experiments.

3.5.1. *Escherichia coli*

The viable count of *E. coli*, increased from 5.64 log cfu/ml to 8.56 log cfu/ml after 24 h of incubation in the control (no preservative added). The effect of CGC/chitosan was found to be concentration dependent. CGC/chitosan, when added at a concentration of 0.01%, decreased the viable counts by 3–4 log cycles in 4 h but after 24 h incubation there was no significant effect on the survival of *E. coli*. All concentrations of CGC/chitosan above 0.01% were bactericidal for *E. coli* (Fig. 6). Other workers have also reported the bactericidal activity of chitosan on *E. coli* (Liu, Guan, Yang, Li, & Yao, 2001; Sudharshan, Hoover, & Knoor, 1992; Zheng & Zhu, 2003). Membrane damage is postulated to be one mechanism by which *E. coli* is killed

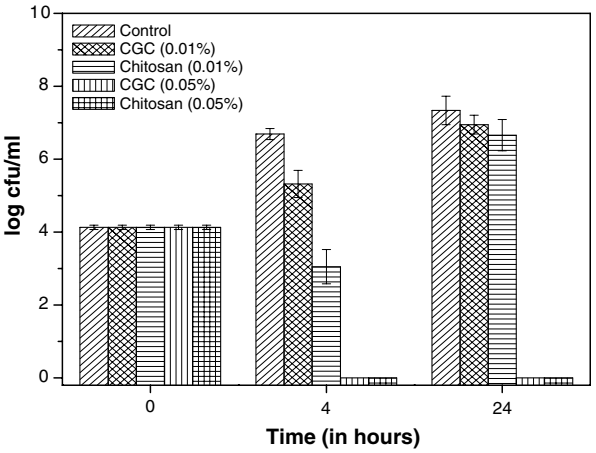

Fig. 9. Antimicrobial activity of CGC/chitosan against *B.cereus* measured in the nutrient broth. At concentrations above 0.01% CGC/chitosan completely inhibited the growth of the test culture. Results are mean values of three independent experiments.

by chitosan (Liu et al., 2001). Polycations of chitosan cross-link with anions on the bacterial surface and this changes the membrane permeability (Tsai & Su, 1999). It has also been reported that the damage of cell membrane by chitosan is concentration dependent and this is in agreement with the findings for bactericidal activity. The noteworthy observation in this study was that the antibacterial effect of CGC was similar to that of chitosan. Conjugation of chitosan with gluten peptides increased the efficiency in inhibiting the growth of *E. coli* cells (Babiker, 2002). Soy protein chitosan conjugate, formed by the Maillard reaction, has also been reported to enhance bactericidal action (Usui et al., 2004).

3.5.2. Pseudomonas spp

The antimicrobial effect of CGC/chitosan against *Pseudomonas* spp is shown in Fig. 7. The results indicated that the antibacterial effect strengthened as the concentration of CGC/chitosan increased. A three-log cycle and two log cycle reduction was observed for CGC (0.01%) and chitosan (0.01%) respectively after 4 h. After 24 h of incubation the *Pseudomonas* count increased to 8.63 and was not significantly affected by 0.01% concentration of CGC/chitosan. At a concentration of 0.05% in 4 h CGC/chitosan had three-log cycle reduction while in 24 h it was completely eliminated. Concentrations of 0.1% and 0.2% CGC/chitosan were bactericidal to *Pseudomonas* spp at both 4 h and 24 h. Therefore, in the case of *Pseudomonas* spp the inhibition profile of CGC was similar to that of chitosan. It has been reported that the minimum inhibitory concentrations of chitosan vary widely from 0.01% to 1.0% (Sagoo, Board, & Roller, 2002). The antimicrobial activity of chitosan depends on various factors like deacetylation degree, temperature and molecular weight. The inhibitory activity of chitosan towards Gram negative bacteria like *Pseudomonas* spp has been reported to be due to the interaction of its polycationic molecules with the predominantly anionic components (LPS) of the Gram negative surface, resulting in changes in its permeability (Chung, Wang, Chen, & Li, 2003). Inhibition of mRNA and protein synthesis has also been suggested for the antimicrobial action of chitosan (Sudharshan et al., 1992).

3.5.3. Staphylococcus aureus

Solutions of CGC/chitosan (0.01%) reduced the numbers of *S. aureus*, a Gram positive bacterium, by only one log cycle in 4 h while in 24 h there was no significant effect of CGC/chitosan on the growth of *S. aureus*. *S. aureus* did not survive when incubated for 4 h at concentrations above 0.01% of CGC/chitosan (Fig. 8). Thus the bactericidal effect of CGC/chitosan is concentration dependent. After 24 h, a concentration of 0.01% CGC/chitosan did not have any effect on the growth of *S. aureus*, while higher concentrations totally killed the cells. The minimum inhibitory concentrations of CGC and chitosan for *S. aureus* were the same (0.05%). Chung, Kuo, and Chen (2005) have reported relatively high antibacterial activity against *E. coli* and *S.aureus* for the chitosan–glucosamine derivative, produced using the Maillard reaction as compared with native chitosan. The formation of a film over the surface of the cell membrane, which prevents the nutrients from entering the cell, has been postulated to be the main mechanism for the antibacterial activity of chitosan against *S. aureus* (Zheng & Zhu, 2003).

3.5.4. Bacillus cereus

The effect of CGC/chitosan on the growth of *B. cereus* is shown in Fig. 9. At lower concentrations the antibacterial effect of these compounds was not as strong. At a concentration of 0.01%, the viable counts decreased from 6.69 to 5.32 and 3.05 log cfu/ml with CGC and chitosan, respectively, in 4 h. As the concentration increased to 0.05%, 0.1% and 0.2% the antibacterial effect was reinforced and 100% kill of *Bacillus cereus* was obtained. After 24 h, a concentration of 0.01% CGC/chitosan did not have any effect on the growth of *Bacillus cereus*. At higher concentrations (0.05%–0.2%) of CGC/chitosan, the growth of *B. cereus* was completely suppressed and no viable cells could be detected after 24 h. No, Park, Lee, Hwang, and Meyers, 2002 also found chitosan (0.1%) to be inhibitory to the growth of *B. cereus* isolated from spoiled tofu.

3.6. Effect of CGC/chitosan on shelf life of lamb meat and cocktail pork salami

Control raw lamb meat spoiled in a few days at chilled temperatures. Addition of CGC/chitosan to meat prior to storage increased its shelf life by more than 2 weeks (Fig. 10). This shows that both these compounds, which are natural, can be used in meat formulations as preservatives. They had the ability to reduce the number of spoilage organisms and thereby increase the shelf life. It has been reported that the polycationic nature of MRPs might be

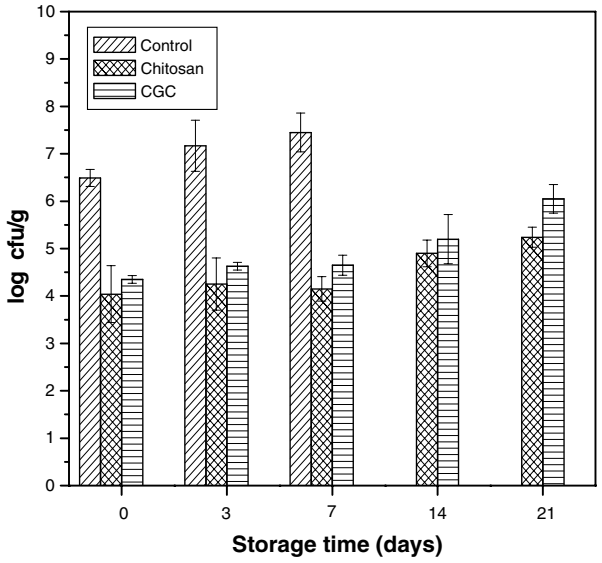

Fig. 10. Effect of Chitosan and MRP on the shelf life of lamb meat stored at 0–3 °C. Results are mean values of three independent experiments.

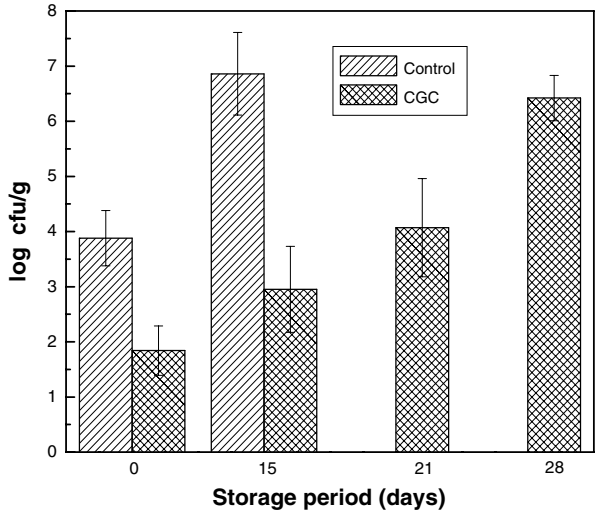

Fig. 11. Effect of CGC on the shelf life of cocktail pork salami store at 0–3 °C. Results are mean values of three independent experiments.

responsible for binding to the anionic cell surface of the microorganisms, resulting in changes in membrane permeability and hence death (Helander, 2001). CGC was then added in the formulation used in the commercial preparation of pork cocktail sausages. The shelf life of salami-containing CGC at chilled temperature was also enhanced as compared to the untreated salami (Fig. 11). Normally the salamis are marketed and stored frozen, as at chilled temperatures they have limited shelf life. However, salami-containing CGC had a shelf life of almost 28 days when stored at 0–3 °C. Thus, in addition to energy conservation a more convenient product (which need not be thawed) could be prepared without any significant change in the sensory properties.

4. Conclusion

It can be concluded from the present studies that the chitosan–glucose complex (CGC) is a better preservative than chitosan alone. It showed superior antioxidant activity as compared to chitosan/glucose alone. The antimicrobial activity of the CGC was identical to that of chitosan against the common food spoilers and pathogens such as *E. coli*, *Pseudomonas* spp, *S. aureus* and *B. cereus*. Thus CGC seems to be a novel natural preservative endowed with both antibacterial as well antioxidant activity and may find applications in the food industry.

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