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Antioxidative Properties of Irradiated Chitosan/Vitamin C Nanoparticles and their Use as Food Additive for Lipid Storage

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CHITOSAN (CS) antioxidant activity improvement was achieved by decreasing their molecular-weight (MW) by γ-rays followed by incorporation with vitamin C (VC) to prepare chitosan/vitamin C (CSVC) complex in the range of nanoparticles. Transmittance Electron Microscopy (TEM) of CSVC complex showed mean diameters ranged from 23.2 to 82 nm.

The antioxidant activities of CSVC complexes were examined using scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and reducing power measurements. CSVC complexes have a synergistic effect on increasing the antioxidant properties rather than their individual effects. The effect of CSVC complexes on lipid peroxidation of meat during 21 days of refrigerated storage was investigated using thiobarbituric acid reactive substance (TBARS) assay.

Treatment of meat with CSVC complex delayed lipid peroxiation about 75% after 7 days of storage as a result the decrease in TBARS values. The results demonstrate promising use of CSVC complex as antioxidants and food additive for lipid storage.

Keywords: Chitosan, radiation, vitamin C, antioxidant, meat storage, lipid.

One of the most important factors that influence the quality and acceptance of meat and poultry during refrigerated or frozen storage is lipid oxidation. The oxidation of lipids leads to rancidity, change in food quality such as colour, aroma, flavour, texture and even the nutritive value of the food. Controlling and monitoring of lipid oxidation during meat processing or storage are important due to the increase demand for precooked convenient meat products for home, fast food and institutional uses (Nissen *et al.*, 2004).

In order to protect lipids, avoid deterioration of appearance, and microbial growth in meat product manufacturers, several food additives with antimicrobial and antioxidant properties were used (Georgantelis *et al.*, 2007, Li *et al.*, 2013 and Suman *et al.*, 2011). The use of food additive in food industry to preserve flavour or enhance its taste and appearance improves food processing, preservation, quality and safety, as well as increased production, cost-effectiveness and sustainability. Nowadays, there is an increased demand for healthier food products without chemical preservatives, resulting in a need to avoid the use of synthetic additives. This has favoured the use of natural additives or alternative methods to extend shelf life and/or improve safety. CS is one of these alternatives. It has been used in food products as preservative in fresh pork sausages (Soultos *et al.*, 2008), fresh pork burgers (Sayas-Barberá *et al.*, 2011), preservative for meat and meat products (Suman *et al.*, 2011), frozen beef burgers (Georgantelis *et al.*, 2007) and edible coatings for fruit and vegetables (Ma *et al.*, 2013) due to its antibacterial and antifungal activities (Rao *et al.*, 2005).

CS has attracted attention as a biomedical material, owing to its unique biological activities including antitumor activities (Suzuki, *et al.*, 1986), immune-stimulating effects (Jeon and Kim, 2001), antimicrobial effects (Park *et al.*, 2004), antioxidant activity (Abd El-Rehim *et al.*, 2012, Sun *et al.*, 2011, and Ying *et al.*, 2011), wound healing effects (Porporatto *et al.*, 2003), free radical scavenging activities (Anraku *et al.*, 2008) and chelating activity that selectively binds protein and metals (Yen *et al.*, 2008). The applications of CS in food industry and medicine are limited because of its high MW resulting in its low solubility in aqueous media (Ilyina *et al.*, 2000). It is important to improve the water solubility of CS to expand its usefulness in food industry. In recent years, radiation induced degradation of CS to obtain low MW and to become more interesting for development of many successful applications in agriculture, health care, food, and environmental protection.

Antioxidants are an important group of food additives as health protecting factors for prevention of oxidative damage and extend shelf life of foods. Recently, the antioxidant activity of CS and its derivatives has attracted attention (Park *at al.*, 2004, Sun *et al.*, 2011, and Ying *et al.*, 2011). Ascorbic acid is a naturally occurring organic compound with antioxidant properties and

can be used as antioxidant and food additives due to the presence of the enediol moiety. The biochemical functions of VC such as scavenger reactive oxygen species, antivirus and antitumor are of increasing interests.

In this study, antioxidant activity of CS was enhanced by subjecting CS to γ-rays at different doses to prepare low MW followed by chemical treatment with VC to obtain CSVC complexes and their effectiveness in reducing the lipid peroxidation during refrigerated storage of meat will be examined.

Materials and methods

Materials

CS, Aldrich, high MW $1.9x10^6$ Da, DD> 85%. VC, El-Nasr Company for chemicals, Egypt. DPPH was purchased from Sigma Alderch, USA. Ferric chloride and ferrous chloride were supplied from BDH. Potassium ferricyanide was supplied from Riedel laboratory reagents. Trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were supplied from SUVCHEM laboratory chemicals. Other reagents and solvents were of analytical grade.

Characterization and analysis

The absorbance was measured by a UV-Vis spectrophotometer JASCO V-560 Japan, in the range of 190-900 nm. The transmittance (%) was measured using infra-red spectrophotometer FT-IR 6300 JASCO, Japan. The MW was determined by Gel permeation chromatography (GPC) 1100 Agilent instrument. The mean diameter and surface distribution were observed by Transmission Electron Microscopy (TEM; JEOL JEM-100CX, Japan). For TEM observations, the CSVC complex solution was properly diluted and dripped onto carbon-coated copper grid and then dried at room temperature.

Preparation of different MWs of CS by exposure to γ-rays

Different MWs of CS were prepared by exposure to γ-rays according to the previous reported method (El-Sawy et al., 2010). Briefly, CS was irradiated at different doses of 25, 50 & 100 kGy by 60Co γ-rays in solid form at dose rate of 3.52 kGy/ h to prepare different MWs of CS and named as CS25, CS50 and CS100.

Synthesis of CSVC complexes

CSVC complexes were synthesized through the reaction between the amine groups of glucosamine units of different MW of CS backbone and VC.

Briefly, certain amount of unirradiated CS (CS0), CS25, CS50 or CS100 was dissolved in 100 ml containing 1% acetic acid then VC solution (the concentration of VC is equal to the concentration of glucosamine unit of CS) was added drop wise into CS solutions with stirring for 4h. The product was precipitated by acetone, filtered, washed with acetone again to remove unreacted compounds, and then dried to obtain different types of CSVC complexes according to the value of CS irradiated dose; CS0VC, CS25VC, CS50VC and CS100VC.

Determination of scavenging activity (%)

Measurement of scavenging activity (%) on DPPH radicals was determined according to the method described previously (Yamaguchi *et al.*, 1998). Briefly, 1.5 ml of DPPH solution (0.1 mM, in 95% ethanol) was incubated with 1.0 ml of different concentrations of CSVC complex solutions. The reaction mixture was shaken well and incubated for 15 min at room temperature and the absorbance of the resulting solution was read at 517 nm against a blank. Ascorbic acid was used for comparison as antioxidant materials. The scavenging activity (%) was measured as a decrease in the absorbance of DPPH and can be calculated using the following equation:

$$\text{Scavenging activity (\%)} = [1 - (A_{\text{samples 517nm}} / A_{\text{control 517 nm}})] \times 100$$

Determination of reducing power

The reducing power was determined by the method described by (Yen and Duh, 1993). Briefly, 1.0 ml of different concentrations of CSVC complex solutions was mixed with 2.5 ml of 0.2 M sodium phosphate buffer pH 6.6 and 2.5 ml of 1% (w/v) potassium ferricyanide. The mixtures were incubated for 20min at 50 °C. The reaction was terminated by adding 2.5 ml of 10% TCA to the mixtures, followed by centrifugation for 10min at speed of 1500 rpm. 2.5 ml supernatant was mixed with 2.5 ml distilled water and 0.5 ml ferric chloride (0.1 %) solution and the absorbance was measured at 700 nm. Increasing the absorbance of the reaction mixture indicates the increase in reducing power of the samples. Ascorbic acid was used for comparison as antioxidant materials.

Preparation of dipping solutions for meat treatment

Aqueous solutions of CS0, VC, CS0VC, CS25VC, CS50VC, and CS100VC were prepared at different concentrations of 0.05, 0.1 and 0.2 % and stirred for 1-2h before using for dipping of meat.

Treatment of meat with CSVC mixtures and storage conditions

Fresh meat was obtained from a local market and was ground using a blender. 10g of ground sample were dipped on the prepared solutions for 10min. Samples without any dipping treatment was used as control. The treated meats were then gently drained on a tissue paper, placed in polyethylene bags and stored in the refrigerator at 4°C for 7, 15 and 21 days. Each treatment contained three replicates and the experiment was repeated three times.

Assessment of lipid oxidation of meat

Lipid oxidation was assessed according to the TBARS assay (Vynche, 1970) and on the basis of the concentration of MDA formed during 0, 7, 15 and 21 days of refrigerated storage (4 °C). Triplicate 10 g samples (for each group) were homogenized with 50 ml of 7.5 % TCA solution. The homogenate was filtered and 5.0 ml aliquot was transferred to a clean screw capped tube and mixed with 5.0 ml of freshly prepared 0.02 M TBA solution. The mixture was put in a boiling water bath for 35 min until colour formation, and then it was left to cool. The absorbance of the developed pink colour was measured at wavelength 532 nm against blank. MDA is one of the aldehydes formed during lipid oxidation in the meat and was expressed as mg MDA/kg sample.

The decrease (%) in MDA/kg at storage time (day)= [($Abs_{at\ 532\ nm}$ of treated samples – $Abs_{at\ 532\ nm}$ of control samples)/ $Abs_{at\ 532\ nm}$ of control samples]x100.

Data analysis

An average value of the replicate analyses was used in calculations of sample variation and significance testing. All statistical analysis was performed with SPSS (SPSS Inc., USA). Values are presented as means.

Results and Discussion

Synthesis and characterization of CSVC complexes

The formation of CSVC complex was carried out by ionic interaction between amino groups of CS and the acidic hydroxyl group at carbon 3 of VC. FT-IR spectra of CS (Fig. 1, curve a) shows basic characteristic absorption bands at 3440 cm^{-1} (O-H and N-H stretch), 1651 cm^{-1} corresponding to the stretching of amide C=O, 1598 cm^{-1} (N-H bend), 1387 cm^{-1} (Amide), 1154 cm^{-1} (asymmetric bridge-O-stretch) and 1089 cm^{-1} (skeletal vibration involving the

C-O stretch). FT-IR of irradiated CS was reported in our previous paper (Abd El-Rehim *et al.*, 2012), it was found there is no significant change between FT-IR of unirradiated and irradiated CS indicating that the stability of the β-glycosidic bonds and distribution of glycosidic bonds in the molecular chains of CS. FT-IR spectra of VC (Fig. 1, curve b) shows four peaks at 3527, 3413, 3317 and 3217 cm^{-1} were attributed to the four –OH groups at C(6)-OH, C(3)-OH, C(5)-OH and C(2)-OH, respectively. The bands at 1754 and 1673 cm^{-1} are corresponding to lactone C=O and C=C stretching, respectively. The C-H stretching is assigned at 3029-2920 cm^{-1} (Lohmann *et al.*, 1984). The bands at 1386 and 1260 cm^{-1} are due to C-O stretching vibration.

FT-IR spectrum of CS0VC (Fig. 1, curve c) shows that the bands at 3440 and 1598 cm^{-1} characteristic of NH_2 bending vibrations gradually weakened and a new absorption band appeared at 1757 cm^{-1} due to C=O group of ascorbic acid. These results suggest that the NH_2 groups in the CS chains were protonated by the H^+ supplied by ascorbic acid (Zheng *et al.*, 2008). The FT-IR spectra of CS25VC, CS50VC and CS100VC (Fig. 1, curves d, e and f) show a decrease in peak intensity at 3440cm^{-1} indicated the reduction of free $–NH_2$ groups after the formation of CSVC mixtures. This is due to radiation degradation of CS lead to decrease inter- and/or intra-molecular hydrogen bonding between the –OH and $–NH_2$ groups resulting in increasing carbonyl groups formation at 1749 cm^{-1} and to increase the amount of functional groups formed such as –OH groups and so the intensity of the band at 3340cm^{-1} corresponding to –OH and $–NH_2$ groups. The vibrational band at 1100 cm^{-1} that corresponds to the ether bond in the pyranose ring has no significant change during the reaction with VC. Fig. 2A shows the MW of CSVC complex decreases with decreasing the starting MW of CS. The MW of CS at 0, 25, 50 and 100 kGy was $1.9x10^6$, $7.8x10^5$, $3.1x10^5$ and $9.7x10^4$ Da, respectively. The MW of the obtained CSVC complexes was $1.68x10^5$, $1.1\ x10^5$, $7.3\ x10^4$ and $4.6\ x10^4$ Da, respectively. Fig. 2B shows UV spectra of VC exhibits a strong absorption band at about 265 nm which is due to the л-л* excitation of the C(2)=C(3) double bond. UV spectra of CS shows absorption band around 280–315 nm which may be caused by the $n\rightarrow\sigma^*$ transition for the amino groups of CS and may also assigned to the n→л* transition for the carbonyl groups. UV spectra of CSVC complex show a strong absorption band at 265 nm and its intensity increases with decreasing the MW of starting CS.

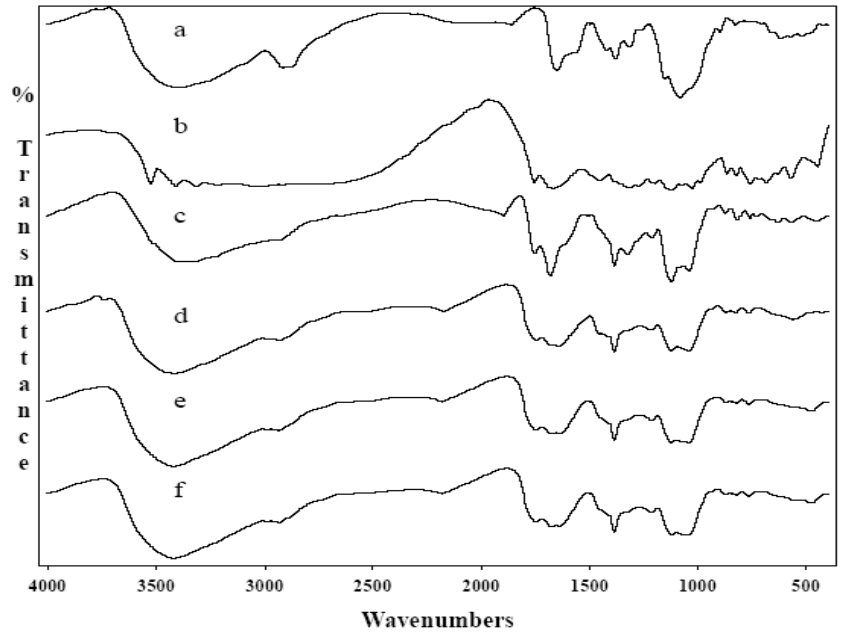

Fig. 1. FT-IR spectra of (a) CS, (b) VC, (c) CS0VC, (d) CS25VC, (e) CS50VC and (f) CS100VC.

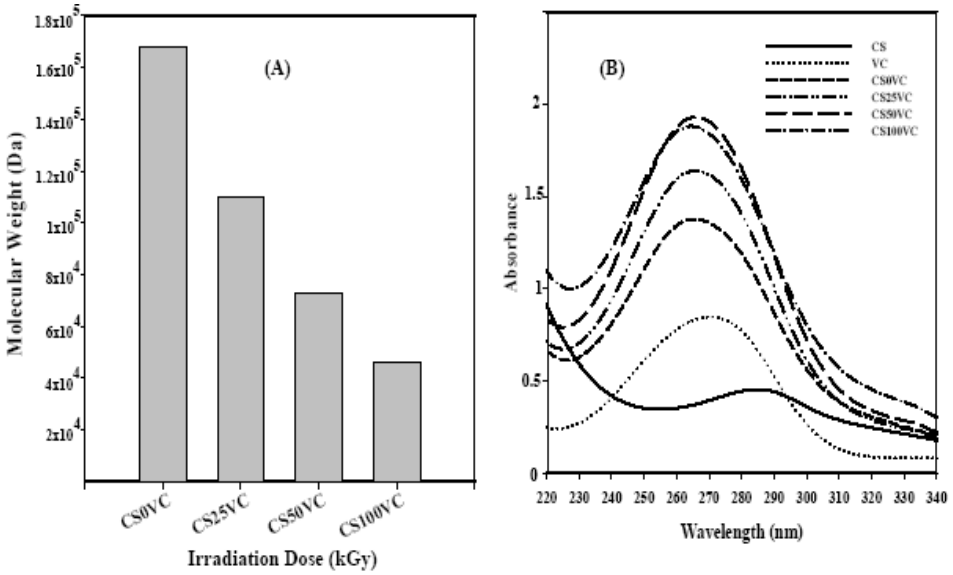

Fig. 2. (A) The Mw of CSVC complexes measured by GPC technique. (B) UV spectra of CS, VC and CSVC complexes.

Fig. 3 shows the morphology of (a) CS50VC and (b) CS100VC complexes analyzed by TEM. The CSVC complex show a dark, solid and consistent structure resulted in aggregated with diameters ranged from 23.2 to 82 nm. These CS50VC mixtures possessed a mean particle size of 82, 52.6, 46.5, 40 and 23.2 nm. Also, CS100VC complexes possessed a mean particle size of 55.4, 48.3, 42.1, 41.1 37.2, 31.5 and 28.2 nm. It was found that particle sized of CSVC complex was controlled by decreasing the MW of starting CS.

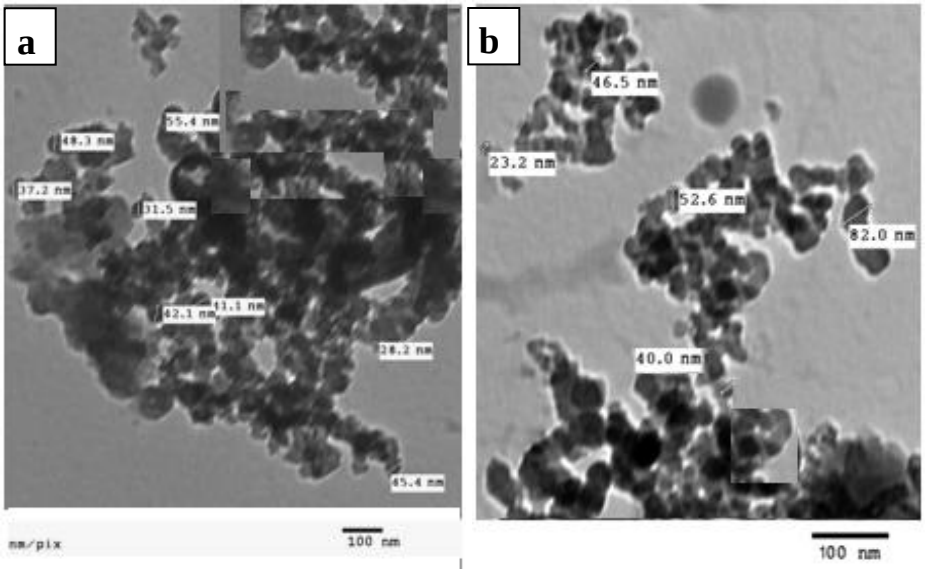

Fig. 3. TEM images of (a) CS50VC and (b) CS100VC.

Scavenging Activity (%)

Antioxidant properties, especially radical scavenging activities, are very important due to the deleterious role of free radicals in foods and in biological system (Pan *et al.*, 2008). Fig. 4. shows the scavenging activity (%) of unirradiated CS, irradiated CS, VC and different types of CSVC complexes on DPPH radicals. Generally, the scavenging activity (%) on DPPH increases with increasing the concentration. The scavenging activity (%) of CS was enhanced by γ-irradiation. The lower MW of CS, the higher scavenging activity. At 70 μg/ml concentration, the scavenging activity (%) of CS irradiated at 0, 25, 50 and 100 kGy was 3, 14.5, 19.8 and 28.5 %, respectively if compared with that of VC (42 %). This is due to the high MW of CS which has compact structure,

thus making the overall effect of their intra-molecular hydrogen bonds stronger lead to decrease the reactivity of hydroxyl and amino groups. On the contrary, low MW CS has a less compact structure, thus making the overall effect of intra-molecular hydrogen bonding less effective and so increase the reactivity of hydroxyl and amino groups.

Incorporation of CS molecules with VC had a synergistic effect on increasing the scavenging activity (%) on DPPH rather than their individual effects. The CSVC complexes with lower MW of CS have a promising effect on increasing the scavenging activity. The CS100VC had the highest scavenging activity on DPPH. At the concentration 70 µg/ml, the scavenging activity (%) on DPPH of CS0VC, CS25VC, CS50VC and CS100VC was 49.2, 72.5, 81.3 and 91.5 %, respectively. Percentage of inhibition IC_{50} % is used very frequently as parameters characterizing the antioxidant power. IC_{50} of CS25VC, CS50VC and CS100VC was 32, 22.5 and 18 µg/ml, respectively. These results revealed that the prepared CSVC complexes have a good antioxidant activity.

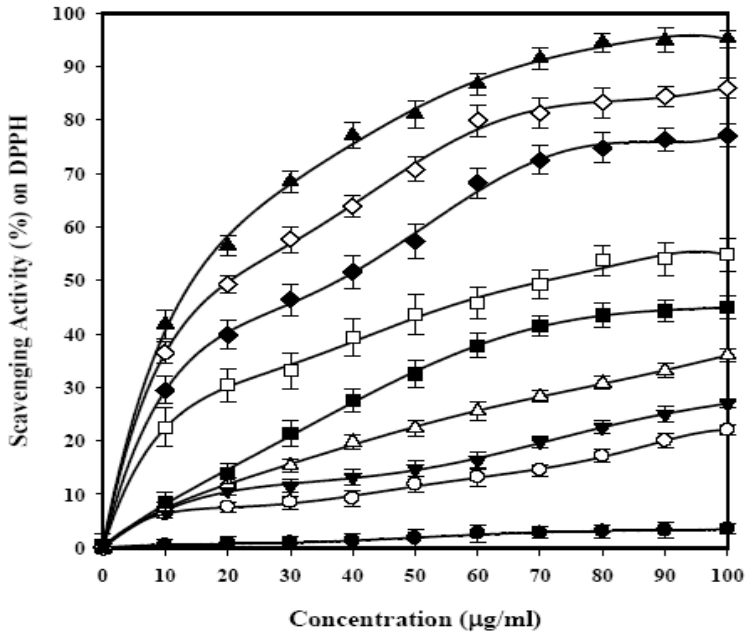

Fig. 4. Scavenging activity (%) on DPPH radicals of (●) CS, (○) CS25, (▼) CS50, (Δ) CS100, (■) VC, (□) CS0VC, (♦) CS25VC, (◊) CS50VC and (▲) CS100VC.

Reducing power

Antioxidant activity has been reported to be concomitant with reducing power. Fig. 5. shows the reducing power of unirradiated CS, irradiated CS, VC and different types of CSVC complexes. CSVC complexes with low Mw CS showed high reducing power and the reducing power increases with increasing the concentration. The reducing power reach its maximum value at 1.0 mg/ml concentration then levelled off with further increase in concentration.

At 1.0 mg/ml concentration, the reducing power of CS0, CS25, CS50, CS100, VC, CS0VC, CS25VC, CS50VC and CS100VC was 0.3225, 0.495, 0.69, 0.8809, 1.3503, 1.2314, 2.582, 2.813 and 2.8877, respectively. Increasing the absorbance indicates increasing reducing power activity. CS50VC and CS100VC complexes showed high reducing properties compared to CS or VC. The increase in reducing power of CSVC complexes indicates the enhancement of their antioxidant activity suggesting their possible use as antioxidants for preventing flavour changes caused by lipid peroxidation.

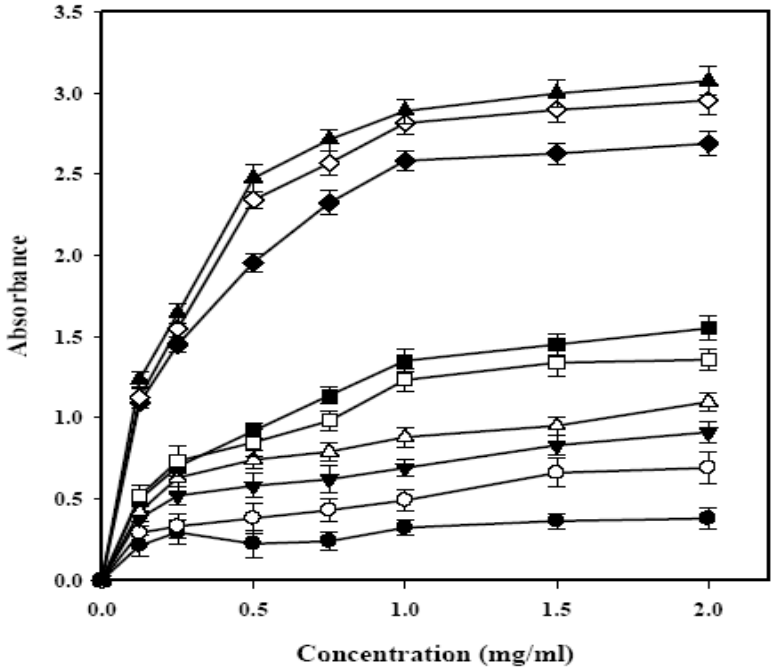

Fig. 5. Reducing power of (●) CS, (○) CS25, (▼) CS50, (∆) CS100, (■) VC, (□) CS0VC, (♦) CS25VC, (◊) CS50VC and (▲) CS100VC.

Lipid oxidation of meat during storage

During storage of meat, lipid peroxides are formed with a subsequent formation of peroxyl radicals, followed by a decomposition phase to yield aldehydes such as MDA (Shahidi, 1994). MDA is an equivalency for stating TBARS values as mg MDA/kg of meat. Table 1. presented TBARS values for treated and untreated meat.

In the present study, control samples (untreated) had the MDA of 0.5042, 0.7481 and 0.955 mg/kg at 7, 15 and 21 days of storage respectively, and would therefore be perceived as rancid already after the 15 days of storage. Whereas TBARS values for meat treated samples were decreased until the end of the storage period. TBARS values changes accordingly to storage period, the type and concentration of the additive. The treatment of meat using CSVC complexes during storage lead to decrease MDA values indicating the decrease in rate of lipid oxidation of meat. The MDA concentration of meat samples treated by CS or VC reached 0.8442 and 0.6823 mg/kg at 21 days and 0.2 % concentration, respectively. Meanwhile, the effectiveness of CS0VC on MDA concentration was 0.5671 mg/kg with a decrease about 32.8-16.8 % in MDA concentration. The incorporation of CS with VC solution improves the protection of the meat samples against lipid oxidation as a decrease in MDA concentration. In comparison with the control and groups of meat treated by CS or VC, TBARS values of CS25VC, CS50VC and CS100VC showed good effect on delaying peroxidation of meat by extending the induction period as results of their antioxidant properties. However, the TBARS value of the CS100VC treated group was higher than the CS25VC and CS50VC treated samples owing to the decrease in MW of CS.

From Table 1, using CS100VC complex at 0.05 % concentration and storage time of 7, 15 and 21 days, the decrease (%) in MDA/kg of meat was 55, 39 and 40.7 %, respectively. While, at 0.1 % concentration the decrease (%) in MDA/kg was 67, 43 and 45 %, respectively, and at 0.2 % concentration the decrease (%) was 75, 46, and 50 %, respectively.

After storage time of 7, 15 and 21 days, the decrease (%) in MDA/kg of treated meat at 0.05 % concentration was 3, 5.3 and 9.8 %, respectively for using CS and was 17.5, 21 and 23.5 % for using VC. While, at 0.2 %

concentration the decrease (%) was 7.5, 8.3, and 11.6 %, for using CS and was 19.6, 21, 28.5 %, respectively for using VC. The increase in the properties and activities of CSVC complex promote their possible use as antioxidant and a food additive for delaying lipid peroxidation.

TABLE 1. Values of TBARS value of treated meat during 7, 15 and 21 days of storage at 4± 1°C using different concentration of different treatments of CS, VC, CS0VC, CS25VC, CS50VC and CS100VC.

Storage Time	7 days			15 days			21 days		
Conc. Type	0.05%	0.1%	0.2%	0.05%	0.1%	0.2%	0.05%	0.1%	0.2%
Control	-	0.5042 a	-	-	0.7481 a	-	-	0.955 a	-
CS	0.4888 b	0.4884 b	0.466 b	0.6953 b	0.6494 b	0.6354 b	0.8613 b	0.851 b	0.8442 b
VC	0.416 c	0.4006 c	0.405 c	0.6162 c	0.6003 c	0.5902 c	0.73 c	0.7205 c	0.6823 bc
CS0VC	0.3782 d	0.3544 d	0.3374 d	0.5623 d	0.5494 d	0.5315 cd	0.6182 d	0.5838 d	0.5671 cd
CS25VC	0.3396 e	0.3051 e	0.2957 e	0.5157 e	0.5015 e	0.4957 de	0.6059 e	0.5808 e	0.525 d
CS50VC	0.2913 f	0.2248 f	0.2147 f	0.4911 f	0.4832 f	0.4692 f	0.5776 f	0.5752 f	0.4955 d
CS100VC	0.2233 g	0.1663 g	0.1259 g	0.4567 g	0.4249 g	0.4036 f	0.566 g	0.5247 g	0.4761 d
LSD	0.0058	0.0015	0.0055	0.0015	0.0038	0.0039	0.0013	0.0015	0.138

Conclusion

CSVC complexes were synthesized in the range of nanoparticles through the ionic interaction of VC with CS molecules of different MW prepared by exposure to γ-rays. CSVC complexes had a synergistic effect on increasing the scavenging activity (%) on DPPH and high reducing power rather than their individual effects. The treatment of meat by CSVC complexes resulted in a highly significant decrease (%) in MDA/kg after 7 days of storage about 75 %. CS50VC and CS100VC complexes showed high decrease (%) in MDA/kg of minced meat. It can be concluded that CSVC complexes could be used as antioxidants and a food additives for lipid storage.

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الخـــواص المضـــادة للأكســـدة لمتراكـــب الكيتـــوزان المشـعع/فيتـامين ج ذو الجسيمات النانويـة واستخدامه كمـادة مضافة للغذاء لتخزين الدهون

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قسم البحوث الإشعاعية لكيمياء البوليمرات ، المركز القومي لبحوث وتكنولوجيا الإشعاع ، ص.ب. 29 مدينة نصر ، القاهرة.

تم تحسين النشاطية المضادة للأكسدة للكيتوزان عن طريق تقليل وزنه الجزيئ باستخدام الإشعاع الجامي متبوعا بتفاعله و دمجة مع جزيئات فيتامين ج لتحضير متراكب الكيتوزان المشعع/ فيتامين ج (CSVC) في نطاق الجسيمات النانوية. وقد أظهرت نتائج الميكروسكوب الإلكتروني النفاذ (TEM) لمتراكب الكيتوزان المشعع/ فيتامين ج (CSVC) أن متوسط قطره يتراوح ما بين ٢٣.٢ و حتي ٨٢ نانوميتر. كما تم دراسة النشاطية المضادة للأكسدة لمتراكب الكيتوزان المشعع/ فيتامين ج من حيث تأثير الإلتهام على DPPH ذات الشقوق الحرة وقياس قوة الإختزال.

و قد أظهرت النتائج ان لمتراكب (CSVC) تأثير فعال على زيادة الخواص المضادة للأكسدة مقارنة بالكيتوزان او فيتامين ج كل على حده. و تم معالجة اللحوم بالمتراكب المحضر (CSVC) ودراسة أكسدة الدهون عند التخزين لمدة ٢١ أيام عند درجة التبريد (٤ درجة مئوية) باستخدام اختبار حامض الثيوباربتيوريك (TBARS test) و التعبيرعنها بتركيز نسبة تكوين الألدهيد المالونالدهيد (MDA/Kg). و قد وجد أن معالجة اللحوم بهذا المتراكب يؤخر من عملية أكسدة الدهون بنسبة ٧٥% بعد تخزين اللحوم لمدة ٧ أيام عند درجة التبريد.

كما أظهرت النتائج أن لمتراكب الكيتوزان المشعع/ فيتامين ج (CSVC) استخدامات واعدة كمادة مضادة للأكسدة و كمادة مضافة للغذاء لتقليل أكسدة الدهون أثناء التخزين.