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Chitosan–caffeic acid–genipin films presenting enhanced antioxidant activity and stability in acidic media

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ABSTRACT

The use of chitosan films has been limited due to their high degradability in aqueous acidic media. In order to produce chitosan films with high antioxidant activity and insoluble in acid solutions caffeic acid was grafted to chitosan by a radical mechanism using ammonium cerium (IV) nitrate (60 mM). Genipin was used as cross-linker. This methodology originated films with 80% higher antioxidant activity than the pristine film. Also, these films only lost 11% of their mass upon seven days immersion into an aqueous solution at pH 3.5 under stirring. The films surface wettability (contact angle 105°), mechanical properties (68 MPa of tensile strength and 4% of elongation at break), and thermal stability for temperatures lower than 300 °C were not significantly influenced by the covalent linkage of caffeic acid and genipin to chitosan. Due to their characteristics, mainly higher antioxidant activity and lower solubility, these are promising materials to be used as active films.

1. Introduction

Chitosan films have been used for numerous applications largely due to their biological properties (Agulló, Rodríguez, Ramos, & Albertengo, 2003; Rhim & Ng, 2007). Furthermore, chemical and enzymatic modifications of chitosans, due to the presence of the amino group, allow preparing functional derivatives with improved physical and biological properties for different fields of application (Dutta, Ravikumar, & Dutta, 2002; Mourya & Inamdar, 2008). Chitosan films are excellent vehicles for incorporating a wide variety of compounds, for example antioxidants, enzymes, vitamins, minerals, and other nutrients (Agulló et al., 2003; Park, Daeschel, & Zhao, 2004; Park, Stan, Daeschel, & Zhao, 2005; Park & Zhao, 2004; Shahidi, Arachchi, & Jeon, 1999).

Chitosan can be modified via a variety of chemical modifications with graft copolymerization being one of the most versatile methods (Jenkins & Hudson, 2001; Mourya & Inamdar, 2008; Zohuriaan-Mehr, 2005). Ammonium cerium (IV) nitrate (CAN) is one of the most used reagents for vinyl graft onto chitin/chitosan (Zohuriaan-Mehr, 2005). Cerium in its tetravalent state is a versatile oxidizing agent, allowing to assume a redox grafting mechanism in two steps: (1) the complex formation of Ce (IV) with the primary amine and the hydroxyl group at the C-3 position and (2) grafting initiation by radicals produced from the complex dissociation (Jung, Chung, & Lee, 2006; Mourya & Inamdar, 2008; Zohuriaan-Mehr, 2005).

Since oxidation is a major problem affecting food quality and biological applications, research has been conducted regarding the improvement of antioxidant activity of chitosan-based polymers by incorporating natural antioxidants such as phenolic compounds (Aytekin, Morimura, & Kida, 2011; Cho, Kim, Ahn, & Je, 2011; Jung et al., 2006; Mathew & Abraham, 2008; Mourya & Inamdar, 2008; Rivero, Garcia, & Pinotti, 2010; Shiu et al., 2010; Siripatrawan & Harte, 2010; Sousa, Guebitz, & Kokol, 2009). Phenolic compounds are known to have antioxidant properties mainly because they can act as free-radical scavengers since the hydroxyl groups can donate an electron or hydrogen atom to a free radical (Dai & Mumper, 2010). On the other hand, chitosan has the ability to chelate metal ions involved in catalysis of oxidative reactions. Therefore, the introduction of phenolic groups into the chitosan structure allows obtaining a new matrix with both types of antioxidant properties (Agulló et al., 2003; Mourya & Inamdar, 2008).

The use of chitosan films has been restricted due to their inherent water susceptibility and relatively low stiffness and strength, especially in moist environments or acidic media, because of the amine groups protonation (Dutta et al., 2002; Mourya & Inamdar, 2008). The formation of stronger and more extensive

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intermolecular associations is possible through chemical cross-linking processes (covalent bond), which improve the physical properties of chitosan films. In particular, cross-linking can be used to enhance mechanical strength and chemical stability, to control aqueous permeability and solubility, and to decrease the aqueous swelling features of chitosan-based films maintaining their biological properties (Khurma, Rohindra, & Nand, 2005; Shahidi et al., 1999). Genipin has been successfully used as an effective cross-linking agent for polymers containing amino groups, such as chitosan (Butler, Ng, & Pudney, 2003; Muzzarelli, 2009). Genipin cross-linked chitosan films exhibit a slower degradation rate and, in addition, this compound revealed to have 4 orders of magnitude less cytotoxicity than glutaraldehyde, the mostly used cross-linker for chitosan (Jin, Song, & Hourston, 2004; Mi et al., 2006).

The aim of this study was to develop a methodology to prepare chitosan-based films with enhanced antioxidant activity and insoluble in acidic media. In order to improve the antioxidant activity of the chitosan, caffeic acid molecules were grafted to the glucosamine residues of chitosan, due to the high antioxidant capacity of this phenolic acid (Sato et al., 2011). Genipin, a cross-linker, was also added to the chitosan to decrease the solubility of the films in acidic media. The antioxidant activity, solubility, surface and mechanical properties, and thermal stability of the films were determined in order to evaluate if the developed films would have potential to be used as active polymers for application in acidic media.

2. Materials and methods

2.1. Materials

Chitosan of medium molecular weight with a degree of deacetylation of 85% (according to the producer) and caffeic acid (≥98% purity) were supplied by Sigma–Aldrich (St. Louis, MO, USA). Ammonium cerium (IV) nitrate (CAN) with ≥98% purity was obtained from BDH (London, UK). Genipin with ≥98% purity was acquired from Challenge Bioproducts Co. (Taiwan, China). All other reagents used were analytical grade.

2.2. Film preparation

Chitosan solution was prepared by dissolving 1.5% (w/v) chitosan in 5% (v/v) acetic acid aqueous solution, with stirring for 16 h at room temperature. 50 g of chitosan solution was added to 50 mL of aqueous solution of ammonium cerium (IV) nitrate (CAN) with the concentration of 6, 30, 60 or 90 mM and 4 mL of 4% (w/v) caffeic acid in ethanol. This mixture was kept under nitrogen atmosphere at 40 °C, in the dark, for 3 h, with stirring. At the end of the reaction 600 mL of distilled acetone was added to the mixture in order to precipitate the modified chitosan. The precipitate was obtained by centrifugation at 24,600 × *g* for 20 min at 4 °C. The precipitate was washed with 100 mL of methanol, during 1 h with stirring, to remove the caffeic acid not covalently bound to chitosan. After centrifugation, the precipitate was dissolved in 45 mL of acetic acid 5% (v/v) and, after complete dissolution, 0.4 g of glycerol were added. This mixture was placed in a water bath at 50 °C with stirring for 10 min. After cooling to room temperature the solution was filtered under vacuum through a porous glass filter (G2) and degassed. This solution (31 g) was transferred into a plexiglass plate with 144 cm^2 with 3 mm deep and was placed in an oven for 16 h at 35 °C for film formation by solvent casting. Chitosan films (Ch) were also prepared using the same methodology, except by the addition of the CAN and caffeic acid solutions.

To prepare the films of chitosan cross-linked with genipin (Ch-Ge) and the films of chitosan grafted with caffeic acid and cross-linked with genipin (Ch-CA-Ge), after the addition and homogenization of glycerol, 250 μL of 10% (w/v) genipin in ethanol was added to the mixture. The mixture was homogenized with constant stirring for 30 min. The solution was then filtered, degassed, and transferred to a plexiglass plate as described above. After 6 h of genipin addition, the plates were placed in the oven for 16 h at 35 °C for film formation.

The films (Ch, Ch-CA, Ch-Ge, and Ch-CA-Ge) were washed with methanol in a Soxhlet extractor for 2 h (12 cycles/h) to extract all the caffeic acid and genipin not covalently linked to chitosan.

All films prepared were neutralized by immersion in 1 M NaOH for 1 h. The films were then thoroughly washed with distilled water until pH 6. These neutralized films were left to dry at room temperature.

The linkage of chitosan to caffeic acid and genipin was confirmed by FTIR analysis. The yield of grafting, determined by the relative increase of the antioxidant activity of the Ch-CA films using the ABTS method comparing with the antioxidant activity of Ch films and a standard of caffeic acid (Aytekin et al., 2011), was less than 0.1%. The cross-linking yield, estimated by the difference between the content of primary amine groups of Ch films and Ch-Ge or Ch-CA-Ge determined by the ninhydrin colorimetric method (Mi, Shyu, & Peng, 2005), was below the quantification limit of the method (25 μg/cm^2).

2.3. Films characterization

2.3.1. Antioxidant activity

The antioxidant activity of the films produced were determined by an adaptation of the method of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), ABTS, described by Re et al. (1999). A solution of 7 mM ABTS was prepared in 2.45 mM potassium persulfate. This solution was left in the dark, at room temperature, for 12–16 h for $ABTS^{\bullet+}$ formation. 1 mL of $ABTS^{\bullet+}$ was diluted in 80 mL of ethanol and the concentration of the solution was adjusted to obtain an absorbance value at 734 nm between 0.700 and 0.800, using a spectrophotometer (Jenway 6405 UV/Vis).

One square (1 cm^2) of film was placed in 1.5 mL of $ABTS^{\bullet+}$ solution and left to react in the dark with orbital stirring at 80 rpm. The absorbance at 734 nm of the solution was measured after 72 h of reaction. The absorbance of the $ABTS^{\bullet+}$ solution without film was also measured after 72 h (blank). All measurements were performed in triplicate. The antioxidant activity was determined by the percentage of inhibition of the $ABTS^{\bullet+}$ and was calculated as follows:

$$\text{Inhibition ratio}\,(\%) = 100 \times \frac{A_b - A_f}{A_b},$$

where A_b and A_f are the absorbance of blank (without film) and with film after 72 h, respectively.

2.3.2. FT-IR and chemometric analysis

FT-IR spectra of the films were obtained using a Golden Gate single reflection diamond ATR system in a Perkin Elmer Spectrum BX spectrometer. Spectra were recorded at the absorbance mode from 4000 to 600 cm^{-1} (mid infrared region) at a resolution of 8 cm^{-1}. Five replicates (128 co-added scans) were collected for each sample. The obtained spectra were transferred in the JCAMP-DX format and analyzed with a program developed in the Institut National Agronomique Paris-Grignon in collaboration with the University of Aveiro (Barros, 1999). The FT-IR spectral region used for Principal Component Analysis (PCA) was set to 1800–1400 cm^{-1}. Prior to multivariate analysis, the spectra were SNV (standard normal deviates) corrected, i.e., each spectrum was mean centered and divided by the standard deviation.

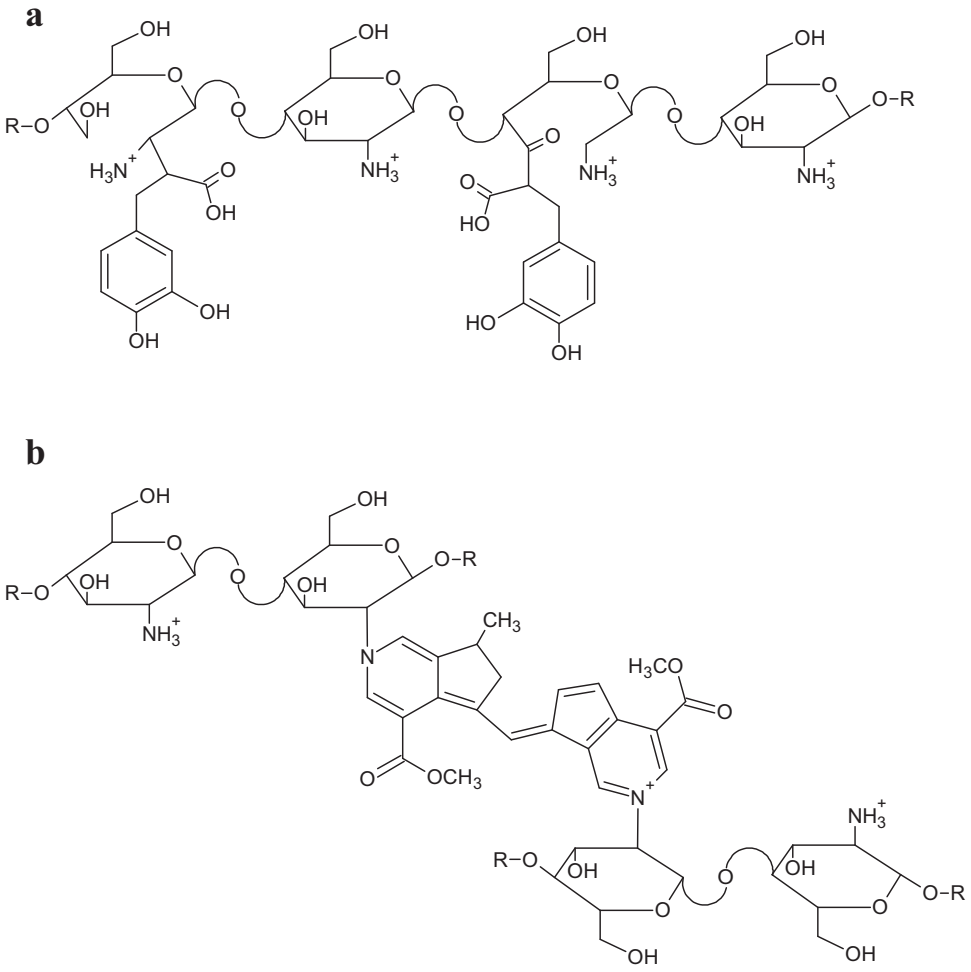

Fig. 1. Proposed structure of chitosan polymer: (a) grafted with caffeic acid and (b) cross-linked with genipin.

2.3.3. Solubility in acid medium

The films solubility was determined in acidic aqueous media (water at pH 3.5 adjusted with hydrochloric acid). One square (4 cm^2) of film was placed in 30 mL of water at room temperature with orbital agitation (80 rpm) for 7 days. Then, films were placed in an oven at 105 °C for 16 h. After cooling down to room temperature, the films were weighed. The solubility was determined by the percentage of weight loss calculated as follows:

$$\text{Weight loss}\,(\%) = 100 \times \frac{m_b - m_a}{m_b}$$

where m_b and m_a are the weight of dry film before and after being immersed in water at pH 3.5, respectively. This determination was performed in triplicate.

The films moisture was determined by measuring their loss of weight, upon drying in an oven at 105 °C until reaching a constant weight (dry film weight). Samples were analyzed at least in triplicate.

2.3.4. Water contact angle

The measure of the static water contact angle on the surface of each neutralized films (Ch, Ch-Ge, Ch-CA, and Ch-CA-Ge) was performed using a contact angle measuring system (OCA 20, Dataphysics) at room temperature. A drop of 3 μL of ultrapure water was dispensed on the surface of each film (1 × 10 cm) using a microsyringe. The contact angles of the drops were calculated by an image analysis software (dataphysics SCA20_M4) using the Laplace–Young method. Ten droplet images were obtained for each film surface.

2.3.5. Mechanical properties

The mechanical properties of all neutralized films (Ch, Ch-Ge, Ch-CA, and Ch-CA-Ge) were evaluated as described by Santos, Seabra, Veleirinho, Delgadillo, and Lopes da Silva (2006). The tensile test was performed at room temperature using a texture analyser equipment (model TA.Hdi, Stable Micro Systems) equipped with fixed grips lined with thin rubber on the ends. All films were cut in strips with 90 mm length and 10 mm wide for the determination of tensile properties. The film ends were mounted on the grips and the initial grip separation was set at 50 mm. The crosshead speed was set at a constant rate of 0.5 mm/s. Young's modulus (E), percentage elongation or strain at break (ε_b), and tensile strength or stress at break (σ_b) were determined from stress–strain curves obtained from uniaxial tensile tests to film failure. These parameters were calculated based on ASTM D 882-83 standard method. At least six samples of each film type were tested.

Film thickness was measured to the nearest 0.001 mm using a hand-held micrometer (Mitutoyo Corporation). Three thickness measurements were taken on each tensile testing specimen along the length of the rectangular strip.

2.3.6. Thermogravimetric analysis (TGA)

The thermal stability and degradation profile of all neutralized films (Ch, Ch-Ge, Ch-CA, and Ch-CA-Ge) was measured by

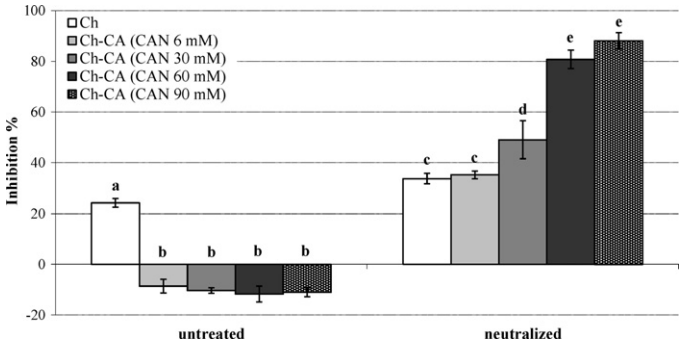

Fig. 2. Antioxidant activity (inhibition %) of chitosan film (Ch) and chitosan films grafted with caffeic acid (Ch-CA) using CAN 6, 30, 60, and 90 mM. Different letters represent values that are significantly ($p < 0.05$) different ($n = 3$).

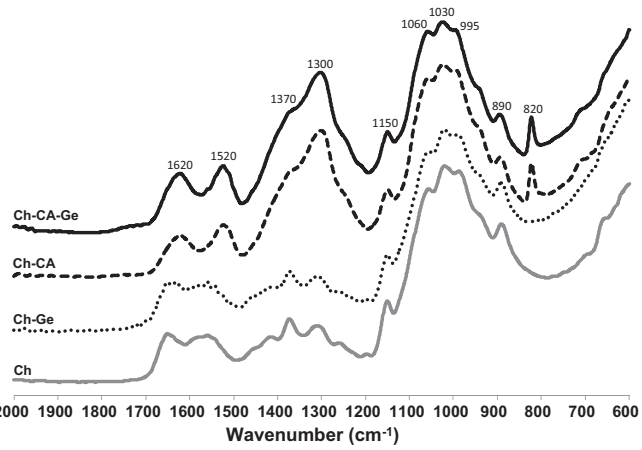

Fig. 3. FT-IR spectra of chitosan (Ch), chitosan cross-linked with genipin (Ch-Ge), chitosan grafted with caffeic acid (Ch-CA), and chitosan grafted with caffeic acid and cross-linked with genipin (Ch-CA-Ge) films.

thermogravimetric analysis (TGA) using a Shimadzu TGA-50 automatic analyzer. Film samples (approximately 4 mg) were heated at a constant rate of 10 °C/min from 50 °C to 600 °C in a dynamic synthetic air atmosphere (20 mL/min).

2.4. Statistical analysis

The results of antioxidant activity and solubility were evaluated statistically in order to determine what or which were significantly different using *F* and *t*-Student tests of Microsoft Excel 2003 with a significance level of 95%.

3. Results and discussion

3.1. Optimization of the concentration of caffeic acid for the preparation of chitosan-based films

In this work caffeic acid was covalently linked to chitosan. This grafted polymer was prepared by generating free radicals on the biopolymer backbone and then allowing these radicals to serve as macroinitiators for the vinyl group on the caffeic acid molecule. Ammonium cerium (IV) nitrate (CAN) was used as it is one of the most used reagents for vinyl grafting onto chitin/chitosan (Zohuriaan-Mehr, 2005). Fig. 1a shows the proposed structure for the chitosan polymer grafted with caffeic acid.

The methodology used to prepare the chitosan-based films grafted with caffeic acid was optimized in order to prepare films with higher antioxidant activity. With this purpose, chitosan-based films were prepared using different concentrations of CAN.

The effect of the CAN concentration used in the preparation of the chitosan films grafted with caffeic acid on their antioxidant activity is shown in Fig. 2 for the films obtained before and after neutralization. Before neutralization with NaOH 1 M (untreated), grafted films revealed pro-oxidant activity, inferred by the negative value obtained for the antioxidant activity. This pro-oxidant activity was probably due to the presence of the oxidant reagent (CAN) used as initiator of the grafting, even though the films were extensively washed by Soxhlet extraction. In order to prove this hypothesis, chitosan films were prepared using CAN in the same conditions as those used for the caffeic acid grafted ones but without addition of caffeic acid. The antioxidant activity of these films revealed also a negative value (data not shown), confirming that it was the presence of CAN in the films that provided their pro-oxidant activity.

The films neutralized by the addition of NaOH and subsequently washed until pH 6 revealed positive antioxidant activity values. As the $ABTS^{\bullet+}$ solutions did not change the pH upon the addition of the films under analysis (data not shown), it can be inferred that the neutralization and the subsequent washing were able to remove the CAN still remaining in the films. In the neutralized films the antioxidant activity increased with the increasing of CAN concentration until 60 mM. The films prepared with 6 mM had the same antioxidant activity of the ones prepared only with chitosan, while the films with CAN 30 mM showed a slight increase of antioxidant activity. The highest antioxidant activity was obtained for the films produced with 60 and 90 mM of CAN (around 80%), which was a value two times higher than the value obtained for the chitosan films. The increase in the CAN concentration to 90 mM did not improve the antioxidant capacity of the films. This result allowed selecting the concentration of 60 mM of CAN to prepare chitosan films grafted with caffeic acid.

In summary, for the preparation of chitosan-based films with higher antioxidant by caffeic acid grafting, 1.5% chitosan, 4% caffeic acid, and 60 mM CAN should be added simultaneously and allowed to react for 3 h at 40 °C. After precipitation of the modified chitosan and its redissolution, the polysaccharide solution can be dried in plates and the films obtained should be treated with NaOH and thoroughly washed. The amount of cerium present in the washed films was determined to be 15 $\mu g/cm^2$, a value very much lower than the oral toxicity referred in literature a LD_{50} of 1–4 g/kg of rat and mice body weight (Gad, 2005).

3.2. Characterization of chitosan-based films

As the purpose of this work was to develop a chitosan-based film with improved antioxidant activity and insoluble in acidic media, the decrease of the solubility of the films was achieved by adding genipin as a cross-linker to the chitosan grafted with caffeic acid (Ch-CA-Ge). These Ch-CA-Ge films were further characterized and compared with the pristine chitosan film (Ch), chitosan cross-linked with genipin (Ch-Ge), and chitosan grafted with caffeic acid films (Ch-CA).

3.2.1. FT-IR spectroscopy

FT-IR spectroscopy was used to confirm changes in chemical structure of the films prepared using the developed methodology, since changes in covalent bonds and chemical interactions can be tracked by modifications in characteristic spectroscopic signals. Fig. 3 shows the FT-IR spectra of Ch, Ch-Ge, Ch-CA, and Ch-CA-Ge films. The larger differences can clearly be observed between films with caffeic acid (Ch-CA and Ch-CA-Ge) and those without caffeic acid (Ch and Ch-Ge), with the main spectral differences at 1200–1500 cm^{-1} and 800–850 cm^{-1}. These bands can be assigned to the presence of the caffeic acid aromatic ring, since —OH in plane

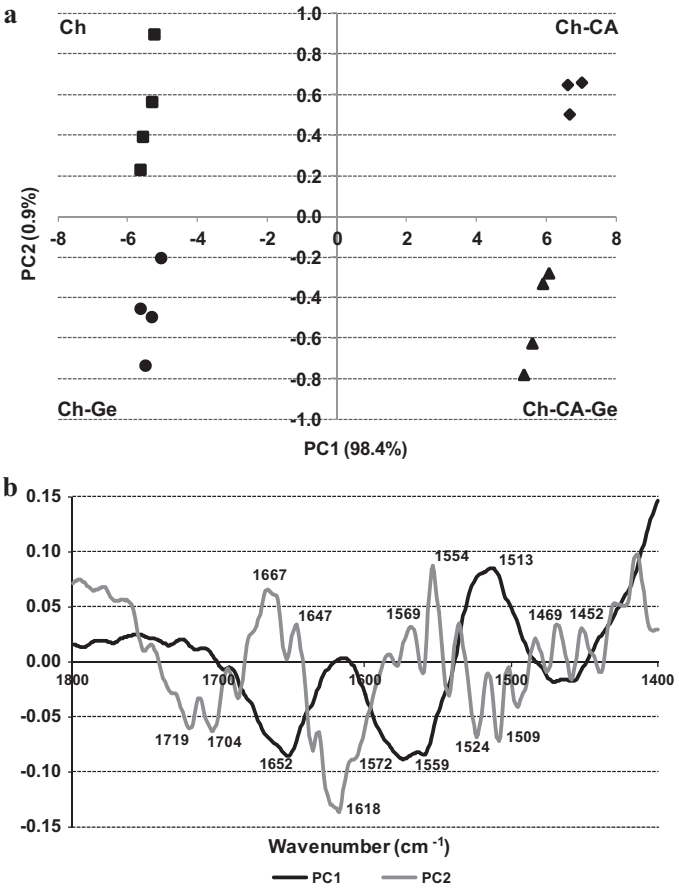

Fig. 4. PCA of the 1800–1400 cm^{-1} spectral region of FT-IR spectra of the different type of chitosan-based films. (a) PC1 vs. PC2 scores scatter plot. (b) PC1 and PC2 loadings profile.

bending of phenolic ring can be attributed to 1365 cm^{-1}, the aromatic ring C=C stretching is within the 1450–1600 cm^{-1}, C—O/C—C stretching vibrations are within the 1200–1300 cm^{-1}, and the C—H bending vibration is between 800 and 900 cm^{-1} (Božič, Gorgieva, & Kokol, 2012; Xu, Uyama, Whitten, Kobayashi, & Kaplan, 2005).

Films spectral region between 1800 and 1400 cm^{-1} was further submitted to chemometric analysis in order highlight spectral differences between the four types of films. Fig. 4a shows the PCA scores scatter plot with PC1 versus PC2. These two principal components explain 99.3% of the information in the FT-IR spectra, being the PC1 responsible for 98.4% and the PC2 only for 0.9%. In this scores scatter plot each film was located in a different quadrant: Ch-CA film in PC1 and PC2 positive region, Ch-CA-Ge film in PC1 positive and PC2 negative, Ch film in PC1 negative and PC2 positive, and Ch-Ge film in PC1 and PC2 negative region.

Discrimination across PC1 axis reinforces the differences previously detected between films with and without caffeic acid. Films with caffeic acid were located in the PC1 positive region and in the negative region were placed the films without caffeic acid (Fig. 4a). PC1 loadings profile shows negative peaks at 1572–1559 cm^{-1} and 1652 cm^{-1} related to non-caffeic acid films (Fig. 4b). These peaks are characteristic of chitosan spectra profile, assigned respectively to the stretching vibration of N—H and C=O of amide of acetylated groups. The signal at ~1580 cm^{-1} was assigned to N—H bond of primary amine groups from chitosan structure (Jung et al., 2006; Martins, Cerqueira, & Vicente, 2012; Pawlak & Mucha, 2003). The PC1 positive peak at 1513 cm^{-1} is correlated to chitosan films with caffeic acid, attributed to the C=C stretching vibration of the

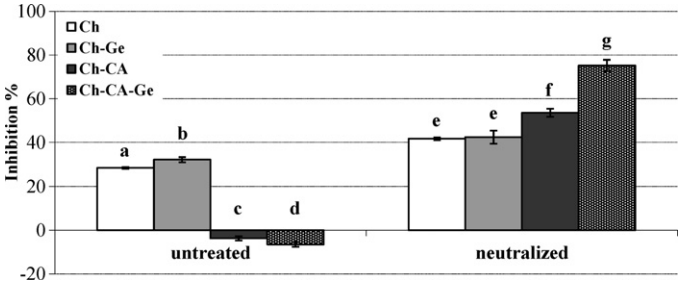

Fig. 5. Antioxidant activity (inhibition %) of chitosan (Ch), chitosan cross-linked with genipin (Ch-Ge), chitosan grafted with caffeic acid (Ch-CA), and chitosan cross-linked with genipin and grafted with caffeic acid (Ch-Ge-CA) films. Different letters represent values that are significantly ($p < 0.05$) different ($n = 3$).

aromatic ring of caffeic acid (Shiu et al., 2010; Xu et al., 2005), confirming the presence of caffeic acid residues in the chitosan films.

PC2 axis allows the discrimination between films with or without genipin. Films with genipin can be found in the PC2 negative region and in the PC2 positive region are placed the films prepared without the addition of genipin (Fig. 4a). PC2 loadings profile shows positive peaks characteristics of chitosan structure (1554 and 1647 cm^{-1}), as described above (Fig. 4b). The negative peaks in PC2 were located at 1509–1524, 1618, and 1704–1719 cm^{-1}. The band at 1509–1524 cm^{-1} can be assigned to the presence of ring-stretching of heterocyclic amine (Butler et al., 2003; Mi, Sung, & Shyu, 2000). The peak at 1618 cm^{-1} is attributed to the C=C stretching from cycloalkene in genipin structure (Mi et al., 2005). The 1719 cm^{-1} signal has been reported to correspond to the C=O stretching vibration due to the ring-opening reaction and the formation of aldehyde groups on the genipin opened ring (Mi et al., 2005). According to these results, the genipin was linked to the chitosan by formation of a heterocyclic compound, as proposed by Butler et al. (2003). In addition, the cross-linking should occur by the linkage between two molecules of genipin bonded to chitosan (Fig. 1b). This mechanism proposed by Chen, Wei, Bisi, Martoni, and Prakash (2005) was confirmed by the presence of the peaks assigned to the N—H and C=O stretching vibrations (~1560 and ~1640 cm^{-1}, respectively). These spectral signals, indicative of the formation of secondary amides as a result of the reaction between the ester groups on genipin with the amino groups on chitosan, are not present in the films cross-linked with genipin (Butler et al., 2003; Chen et al., 2005).

3.2.2. Antioxidant activity

Fig. 5 shows the antioxidant activity of the four films (Ch, Ch-Ge, Ch-CA, and Ch-CA-Ge) under study. A slight increase (13%) of the antioxidant activity was observed for the untreated films with the addition of the cross-linker (Ch-Ge film). Genipin is described as having antioxidant activity (Lee, Lee, & Jeong, 2009). However, the modifications introduced by the cross-linking reaction provide residues that seem to slightly improve the antioxidant capacity of the films. The films with caffeic acid (Ch-CA and Ch-CA-Ge) showed pro-oxidant activity, as was already observed in Fig. 2.

The addition of genipin to the chitosan did not have influence in the antioxidant capacity of the neutralized film. However, the neutralized films with the highest antioxidant activity were the ones with caffeic acid and genipin (Ch-CA-Ge), which were 40% higher than the films with caffeic acid (Ch-CA) and 80% higher than the films made only with chitosan. These results show that cross-linking of chitosan with genipin improves the antioxidant capacity of the films produced with chitosan grafted with caffeic acid. The higher antioxidant activity of these films seems to be due to a synergistic effect on the antioxidant activity of the chitosan-based films by the presence of caffeic acid and genipin, since the increase was

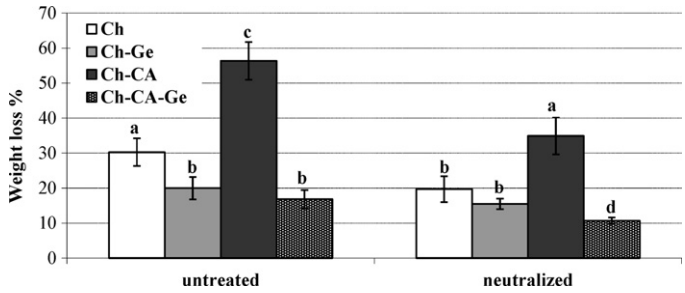

Fig. 6. Solubility (weight loss %) in acidic media (pH 3.5) of chitosan (Ch), chitosan cross-linked with genipin (Ch-Ge), chitosan grafted with caffeic acid (Ch-CA), and chitosan cross-linked with genipin and grafted with caffeic acid (Ch-CA-Ge) films after 7 days with stirring. Different letters represent values that are significantly ($p < 0.05$) different ($n = 3$).

higher than the sum of the antioxidant activity of each compound in the chitosan-based films.

3.2.3. Solubility

Higher degradability in aqueous media is one of the major problems of chitosan-based films (Mourya & Inamdar, 2008). The solubility of films in water may provide insight on the behavior of a film in an aqueous environment, including their degradability or resistance under those conditions.

The films solubility was determined after 7 days immersed in acid water (pH adjusted to 3.5 with hydrochloric acid) under continuous stirring. Fig. 6 shows the weight loss of the four types of films untreated and neutralized. In untreated films, the Ch-CA films showed the highest weight loss (56%). The higher solubility of these films could be due to the decrease in the intermolecular hydrogen bonding between chitosan chains due to the presence of the caffeic acid groups along the chitosan chain (Jung et al., 2006). As expected, a decrease in solubility (30%) was visible for the films with the cross-linker, Ch-Ge and Ch-CA-Ge.

The neutralization of the films decreased their solubility in the acidic medium. The Ch and Ch-Ge films showed a solubility of ~20% of weight loss after 7 days (Fig. 6). The films that exhibited the lowest weight loss (11%) were Ch-CA-Ge. The solubility of these films was almost 70% lower than Ch-CA and 50% lower than Ch films. The stability of these films in acidic media was due to the presence of the cross-linker, the genipin.

3.2.4. Surface properties

The surface wettability of chitosan based films was measured by contact angle analysis using water. The contact angle is an indicator of the hydrophilic/hydrophobic properties of the films. The contact angles of all types of chitosan based films are listed in Table 1. It was observed that there was no significant difference in the surface wettability of Ch, Ch-CA, and Ch-CA-Ge films. Only the Ch-Ge films had a slightly lower value (4%) of contact angle, an effect previously described for films of chitosan cross-linked with genipin (Jin et al., 2004). These results allowed to conclude that although the films surface became more hydrophilic because of the cross-linking, the simultaneous cross-linking with genipin and grafting with caffeic acid had no significant effect on the wettability of chitosan film.

3.2.5. Mechanical properties

The mechanical properties of the four films were studied by uniaxial tensile tests. Table 1 shows the Young's modulus, tensile strength, and elongation at break determined from the obtained stress–strain curves. Young's modulus, related to the elastic behavior of the material under linear stress–strain conditions, was lower for films with caffeic acid (Ch-CA and Ch-CA-Ge). This effect is probably related to the formation of a lower cross-linked network due to the presence of caffeic acid covalently linked to chitosan, which indicates a loss of the rigidity of these films. A slight increase, although not statistically significant, was observed for Ch-Ge when compared with Ch films.

The tensile strength, which is related to the mechanical resistance of the films, was higher for Ch-Ge films. The addition of a cross-linker seems to improve the reorganization of the material, thus increasing the rigidity of the films. The percentage of elongation at break, which indicates the flexibility of the films, was not significantly different for the 4 films under study. However, the Ch-Ge films were those with lower values of elongation at break, which is in accordance with Mi et al. (2006), that reported that chitosan films cross-linked with genipin presented higher mechanical resistance and lower flexibility when compared with those obtained only with chitosan. The caffeic acid addition in chitosan films with genipin (Ch-CA-Ge) decreased their tensile strength comparing with Ch-Ge film, showing that the presence of caffeic acid originates chitosan films more brittle and less stress resistant. A similar trend was reported on chitosan films incorporated with α-tocopherol (Martins et al., 2012).

Ch-CA-Ge films showed identical mechanical properties to the films obtained with chitosan alone. These results show that the mechanical resistance and flexibility of the chitosan films were not significantly altered by the addition of the cross-linker and caffeic acid, probably because of the opposite effects of adding the caffeic acid and the cross-linker.

3.2.6. Thermogravimetric analysis (TGA)

The thermal stability of all types of neutralized chitosan-based films was measured using thermogravimetric analysis. The changes in films weight with the increase of the temperature are shown in Fig. 7a. The derivative of TGA curves, as shown in Fig. 7b, revealed two separate degradation steps for all films, the first one around 290 °C and the second one between 480 °C and 550 °C. The first decomposition step is related with the dehydration, depolymerization, and pyrolytic decomposition of the polysaccharide backbone with vaporization and elimination of volatile products (Martins et al., 2012; Zohuriaan & Shokrolahi, 2004). According to the literature, pyrolysis of polysaccharides starts by a random split of the glycosidic bonds, followed by a further decomposition forming acetic and butyric acids and a series of lower fatty acids, where C_2, C_3, and C_6 predominate (Neto et al., 2005). However, no relevant differences in weight loss of this first step were observed for these films. This allowed to conclude that the caffeic acid and genipin addition had no influence on thermal stability of chitosan films in peak temperature of the above-mentioned thermal events.

The second degradation step may result from the thermal degradation of new cross-linked material formed by thermal cross-linking reactions occurring in the first stage of the degradation process (Tang, Wang, & Chen, 2005) or/and could correspond to the dehydration of the sugar rings, depolymerization, and decomposition of the acetylated and deacetylated glucosamine residues (Mathew & Abraham, 2008). In this second step, the peak was shifted to lower temperatures, from 550 °C to 480–510 °C, for the chitosan films containing caffeic acid. This shift in temperature could be attributed to the decrease of the interactions between chitosan chains due to the presence of the molecules linked to the chitosan. This may originate a decrease in the reactions occurring in the first stage of the degradation process. Films containing caffeic acid (Ch-CA and Ch-CA-Ge) showed another peak which could be associated with aromatic structures decomposition, since they are highly stable due to the resonance of the benzene ring, which results in the decomposition only at higher temperatures, above 400 °C (Pelissari, Grossmann, Yamashita, & Pineda, 2009).

The films produced with genipin and/or caffeic acid showed no differences on thermal stability for temperatures lower than 300 °C,

Table 1
Contact angle and mechanical parameters (Young's modulus, tensile strength, and elongation) values obtained for chitosan (Ch), chitosan cross-linked with genipin (Ch-Ge), chitosan grafted with caffeic acid (Ch-CA), and chitosan grafted with caffeic acid and cross-linked with genipin (Ch-CA-Ge) films.

Film	Contact angle (°)	Young's modulus (MPa)	Tensile strength (MPa)	Elongation (%)
Ch	105.2 ± 2.29[a]	31.5 ± 2.62[a]	65.8 ± 4.06[a]	4.4 ± 0.46[a,b]
Ch-Ge	101.4 ± 2.78[b]	35.3 ± 3.00[a]	82.2 ± 3.14[b]	4.0 ± 0.74[a]
Ch-CA	105.5 ± 1.66[a]	22.1 ± 2.82[b]	63.8 ± 3.89[a]	5.3 ± 0.50[b]
Ch-CA-Ge	105.0 ± 1.83[a]	25.2 ± 2.86[b]	67.6 ± 5.59[a]	4.3 ± 0.97[a,b]

Different superscripts letters, in each column, represent values that are significantly ($p < 0.05$) different ($n = 6$ for mechanical properties and $n = 10$ for contact angle).

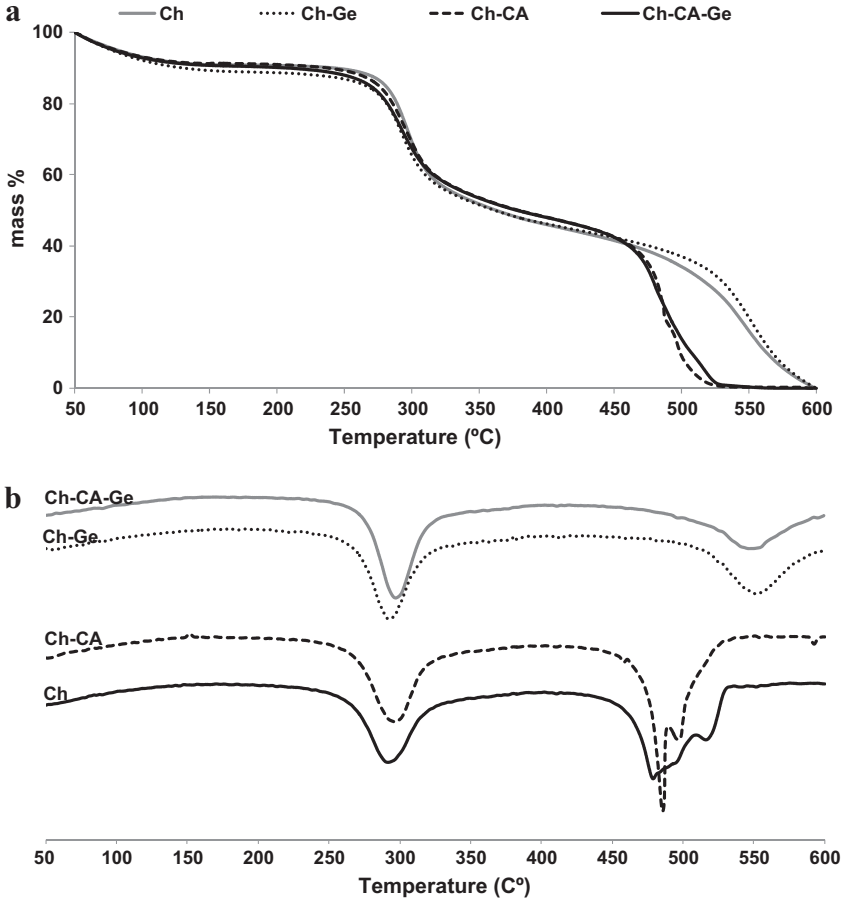

Fig. 7. TGA for chitosan (Ch), chitosan cross-linked with genipin (Ch-Ge), chitosan grafted with caffeic acid (Ch-CA), and chitosan grafted with caffeic acid and cross-linked with genipin (Ch-CA-Ge) films. (a) Weight loss curves of TGA. (b) First derivative of TGA curves.

while for temperatures above 480 °C, lower thermal stability of chitosan films with caffeic acid was observed.

4. Conclusion

This study shows that a film of chitosan grafted with caffeic acid and cross-linked with genipin can be prepared, imparting a good antioxidant activity and low solubility in acid pH. The surface wettability, mechanical properties, and thermal stability of the films were not significantly influenced by the linkage of caffeic acid and genipin to chitosan. Therefore, these films can be promising materials to be used as an active polymer, contributing to biological applications, including food preservation and shelf-life extension.

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