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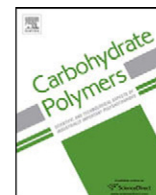

Chitosan–caffeic acid–genipin films presenting enhanced antioxidant activity and stability in acidic media

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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.

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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 $ABTS^{•+}$ formation. 1 mL of $ABTS^{•+}$ 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 $ABTS^{•+}$ 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 $ABTS^{•+}$ 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 $ABTS^{•+}$ 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.

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