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Rheological and antioxidant power studies of enzymatically grafted chitosan with a hydrophobic alkyl side chain

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ABSTRACT

The enzyme-mediated grafting of hydrophobic alkyl side chains onto Chitosan (Ch) has been successfully achieved using octyl gallate and horseradish peroxidase. The properties of the resulting materials have been studied by rheology and electron paramagnetic resonance (EPR) spectroscopy in order to envisage its potential applications as bioactive food additive. The chemical structures of the octyl gallate-grafted Ch were corroborated by ATR-FTIR, ^{1}HRMN, viscosimetric molecular weight and ζ potential. The antioxidant capacity of materials determined by EPR show that functionalized Ch radical scavenging increases with grafting, which was related to the aromatic ring in the octyl gallate. The Ch with the highest grafting exhibited an antioxidant capacity of 81%. Solutions of the materials in acetic acid and lactic acid exhibited a shear-thinning flow behavior which also increased with the grafting. The viscoelastic properties of solutions were characterized by oscillatory shear measurement and the result showed fluid-like viscoelastic behavior. The elasticity of the solutions decreased with the plasticizer addition.

1. Introduction

Currently, there is a growing interest in sustainable polymers particularly in basic applications such as packaging, edible films and coatings which biodegrade under controlled conditions of storage in order to alleviate the growing global synthetic materials waste problem. In this sense, the chitosan and its derivatives have demonstrated great potential as biological packaging material due to its inherent antimicrobial power, film-forming material as well as their versatile physical and chemical properties as several researchers have highlighted in recent reviews (Dutta, Tripathi, Mehrotra, & Dutta, 2009; Vinsová & Vavríková, 2011).

As a result of the interest in this biomacromolecule, a wide range of chitosan-based (Ch) materials have been proposed by means of suitable chemical or enzymatic modifications onto the Ch backbone to improve its solubility in water, antimicrobial properties, flocculant capacity, absorbency and adhesiveness, while remaining biodegradable and biocompatible for potential applications in biomedicine, food or water treatment. Chemical modifications mainly consisted in the insertion of small functional groups such as alkyl, carboxymethyl, saccharides or oligosacharides for increasing the Ch solubility at neutral and alkaline pH without affecting its cationic character (Alves & Mano, 2008; Sashiwa & Aiba, 2004). Other compounds like cyclodextrins, and acids, such as *p*-aminobenzoic, lactobionic, sialic and polyacrylic have also been attached to confer removal capacity of textile dyes, drug delivery, wound healing, inhibition of microorganisms, antioxidant capacity and increasing hydrophilicity (Calero, Muñoz, Ramírez, & Guerrero, 2010; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Liu, Chen, & Pan, 2007; Siripatrawan & Harte, 2010; Xie, Xu, Wang, & Liu, 2002). Pasanphan, Buettner, and Chirachanchai (2010) grafted gallic acid onto Ch by a conjugated reaction with potential use as additive in foodstuffs owing to improved solubility and antioxidant power. Despite of the possibility to modify Ch by chemical means, enzymatic modifications offer alternative routes, especially in products to be applied in foodstuffs due to minimized hazards associated with toxic reagents, in addition to mild and environmentally friendly reaction conditions (Alves & Mano, 2008; Belalia, Grelier, Benaissa, & Coma, 2008; Chao, Shyu, Lin, & Mi, 2004; Curcio et al., 2009; Kumar et al., 2004; Sobahi, Abdelaal, & Makki, 2011; Xiao-Yi, Jian-Ping, Huai-Tian, Gang-Biao, & Ming-Hua, 2011). Research work on enzymatically modified Ch cover those reported by Kumar, Smith, and Payne (1999) who used tyrosinase for

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grafting a wide range of phenolic substrates onto Ch conferring solubility in basic conditions and antioxidant capacity. Vachoud, Chen, Payne, and Vazquez-Duhalt (2001) demonstrated the ability of horseradish peroxidase (HRP) to graft dodecyl gallate (DDG) onto Ch by generating radicals from the phenolic derivative to the reaction media to form stable linkages, mainly between Ch amino moiety and aromatic carbons. Generally, peroxidases have several advantages over tyrosinase as broader substrate range and optimum pH for activity between 5 and 6, which is convenient in Ch modifications as it solubilizes at pH below 6.0.

Gallates are used as antioxidant additives in foods, cosmetics and medicinal preparations as good H donors. Among them, the octyl gallate (OG), unlike DDC and other synthetic antioxidants, has not been pointed as potential cancer precursor. The bulky structure of OG when grafted onto Ch might allow for the reducing of the intra- and intermolecular hydrogen bonding and the alkyl side chain might provide hydrophobicity to Ch molecule associated to thickening properties in the bulk. The improved rheological characteristics might be related with the length of grafted side chains in addition to pH, polymer concentration and external salt concentration (Desbrieres, 2004; Rinaudo, Auzely, Vallin, & Mullagaliev, 2005). Therefore, the solution behavior of modified Ch is essential to predict, design, and characterize in order to envisage its potential of application. In particular, the linear viscoelastic properties of aqueous solutions of Ch are closely related to their colloidal structure while shear rate dependence of viscosity is associated with the structural modifications (Fujita & Kubo, 2002; Vartiainen, Rättö, Lantto, Nättinen, & Hurme, 2008).

However, despite of the aforementioned reports, there is no information regarding the applicability of Ch grafted with hydrophobic alkyl side chains and in the present work, the aim was to study the rheological properties of the enzymatically produced Ch-*g*-OG samples as important parameter linked to the application of this materials in addition to chemical and antioxidant characterizations.

2. Materials and methods

2.1. Materials

Ch (M_v 486.33 kDa, 87.2% deacetylation), OG, horseradish peroxidase (HRP) type II, hydrogen peroxide (H_2O_2), glycerol anhydrous (Gly), lactic acid (L), deuterium oxide (D_2O), methyl alcohol, 1,1-diphenyl-2-picryl-hydrazyl (DPPH•), ninhydrin and ninhydrin reagent were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetone, sodium hydroxide (NaOH), hydrochloric acid (HCl), ethyl alcohol and glacial acetic acid (A), were purchased from J.T. Baker (Phillipsburg, NJ, USA). Deionized water (filtration system Milli-Q) was used for the preparation of all solutions.

2.2. Enzymatic grafting

Ch-*g*-OG was performed following a modification of the method reported by Vachoud et al. (2001). An HCl 2M (pH ~ 2.5) solution of native Ch (1.6% wt/v) was stirred for 1 h. Then, the solution was diluted with 433.3 mL of phosphate buffer (0.1 M, pH 5.5) to obtain a final 0.3% wt/wt Ch concentration, Thereupon, the pH was raised to 4.5 with 1 M NaOH. 100 mL of the Ch solution was mixed separately with 25 mL of two OG solutions in acetone, 10 and 20 mM to obtain a ratio of Ch:OG 80:20 (v/v) and 60:40 (v/v), respectively. HRP (1.4 mg of enzyme/mL phosphate buffer pH 5.5) was added, and the reaction was initiated by adding 600 μL of H_2O_2 and it was sustained with six successive additions of 600 μL of H_2O_2 at 10 min intervals. After the last addition of H_2O_2, the reaction mixture was stirred for 60 min followed by a centrifugation during 20 min at 7500 rpm and 20 °C. The resulted material was kept at −80 °C in an ultra-freezer REVCO (USA) for 24 h, after that, it was lyophilized to obtain the corresponding two materials Ch-*g*-OG1 and Ch-*g*-OG2 as powders. Control experiments were carried out without Ch addition. Products were precipitated in 1/10 v/v of cold methanol as brown powders, filtered and dried in a vacuum oven prior to analyses.

2.3. Characterizations

2.3.1. ^{1}HNMR analysis

Proton nuclear magnetic resonance (^{1}HNMR) spectra were recorded on a Bruker (AVANCE-III 500 (Germany)) instrument at 200 MHz. Ch grafted samples were dissolved in D_2O containing DCl and 3-(trimethylsilyl)-propionic acid was used as internal reference standard. Products from control experiments without Ch were dissolved in deuterated acetone and tetramethylsilane was used as internal reference standard. Deacetylation degree (DD) of Ch was determined according to Hirai, Odani, and Nakajima (1991) and that for grafted samples following equation (1). OG incorporation as molar% was determined according to equation (2).

$$DD\% = \left[1 - \left(\frac{\frac{1}{3}H_{Ac}}{\frac{1}{6}\int H_{2-6} - 2 \times \left(\int Alk_{/15}\right)}\right)\right] \times 100 \quad (1)$$

$$molar\% = \frac{\int Alk_{/15}}{\left[\frac{\int H_{2-6} - 2 \times \left(\int Alk_{/15}\right)}{6}\right]} \times 100 \quad (2)$$

where $\int H_{Ac}$ is the residue of CH_3, $\int H_{2-6}$ is the integration of the massive of Ch and $\int Alk$ is the integration of 6 H of the side alkyl chain of OG.

2.3.2. ATR-FTIR analysis

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra were recorded in a Perkin Elmer ATR-FTIR 100 instrument (Waltham, Massachusetts, USA) at a wavelength range from 650 to 4000 cm^{-1} with 20 scans per sample.

2.3.3. ζ potential

ζ potential values were obtained from Ch-*g*-OG and Ch solutions prepared with 1% w/v of the polymers into a 1% v/v acetic acid solution by stirring during 24 h at room temperature. Solutions were then diluted to 1:10 mL with 1% acetic acid solution and injected into the chamber of a ZetaSizer ZEM5003 (Zetamaster, Malvern Instruments, UK). Smoluchowsky mathematical model was used to convert the electrophoretic mobility measurements into ζ potential values. Measurements were performed by triplicate.

2.3.4. Determination of amino group content by ninhydrin assay

Free amine groups of functionalized Ch were determined according to the method reported by Alonso et al. (2009). 0.1 mg/mL solutions of Ch, Ch-*g*-OG1 and Ch-*g*-OG2 were dissolved in a solution of acetic acid 1% v/v with constant stirring for 24 h at room temperature. Then, 1 mL of ninhydrin reagent freshly prepared, were added to the sample solution. The mixtures were boiled in water for 10 min and transferred to ice-water bath and paper filtered through a Whatman No. 40. Then, the absorbance was measured at 570 nm.