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Functionalization of chitosan by laccase-catalyzed oxidation of ferulic acid and ethyl ferulate under heterogeneous reaction conditions

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ABSTRACT

Chitosan particles were functionalized with ferulic acid (FA) and ethyl ferulate (EF) as substrates using laccase from *Myceliophtora thermophyla* as biocatalyst. The reactions were performed with chitosan particles under an eco-friendly procedure, in a heterogeneous system at 30 °C, in phosphate buffer (50 mM, pH 7.5).

The FA-chitosan derivative presented an intense yellow-orange color stable while the EF-chitosan derivative was colorless. The spectroscopic analyses indicated that the reaction products bound covalently to the free amino groups of chitosan exhibiting a novel absorbance band in the UV/Vis spectra between 300 and 350 nm, at C-2 region by the duplication of C-2 signal in the ^{13}C NMR spectrum, *via* Schiff base bond (N=C) exhibiting novel bands in the FT-IR spectrum at 1640 and 1620 cm^{-1}. Additionally, antioxidant capacities of chitosan derivatives showed that the chitosan derivatives presented improved antioxidant properties, especially for FA-chitosan derivative (EC_{50} were 0.52 ± 0.04, 0.20 ± 0.02 mg/ml for DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging, respectively).

1. Introduction

Chitin is a natural polysaccharide corresponding to the second-most naturally abundant polysaccharide, after cellulose. Chitin is obtained from the shells of crustaceans, the cuticles of insects, the cell walls of the fungi and the yeasts (Mathur & Narang, 1990). Chemically, the polymer skeleton of chitin consists of pyranose rings of β-(1,4)-2-acetamido-2-deoxy-β-D-glucose (*N*-acetylglucosamine, GlcNAc).

The most important chitin derivative is chitosan which consists of pyranose cycles of β-(1,4)-2-amino-2-deoxy-β-D-glucose (*N*-glucosamine (GlcN) is the repeating unit of chitosan) linked with a glycosidic linkage, obtained mainly by deacetylation under alkaline conditions. Such procedure leads to a random distribution of acetylated/deacetylated units along chitosan chains (Khor & Lim, 2003). As a natural renewable resource, chitosan exhibits unique properties such as biocompatibility, biodegradability, non-toxicity, and filmogenicity, as it presents some important applications in the biomedical, agriculture, functional food, wastewater purification, food packaging, and cosmetics domains (Arvanitoyannis & Kassaveti, 2008; Ravi Kumar, 2000; Rinaudo, 2006, 2008). Furthermore, as this polysaccharide exhibits a strong metal ion chelating ability due to its N atoms, it can be used as antioxidant based on metal ion deactivation (Varma, Deshpande, & Kennedy, 2004). However, it presents two main limitations to be considered as a practical antioxidant, especially, (i) its poor solubility due to its inter- and intra-molecular hydrogen bonds network and (ii) the lack of H-atom donors to serve as a good chain breaking antioxidant. Hence, additional groups which could improve this property must be introduced for the development of a chitosan-based antioxidant. It can be expected that the functionalization of chitosan with quinones may enhance its antioxidant activity, antimicrobial activity and antifungal properties against a range of food microorganisms (Hoff, Liu, & Bollag, 1985).

The modification of chitosan by enzyme catalysis has been intensively examined (Chao, Shyu, Lin, & Mi, 2004; Chen, Kumar, Harris, Smith, & Payne, 2000; Lenhart, Chaubal, Payne, & Barbari, 1998; Muzzarelli, Ilari, Xia, Pinotti, & Tomasetti, 1994; Sousa, Guebitz, & Kokol, 2009; Vachoud, Chen, Payne, & Vazquez-Duhalt, 2001; Vartiainen, Ratto, Lantto, Nattinen, & Hurme, 2008). Enzymes may offer clean and safe alternatives to current practices using active starting materials and safe conditions. This enzymatic approach occurs using polyphenol oxidases (PPOs) such as tyrosinases, peroxidases and laccases which convert phenols derivatives

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into quinones. These radicals are active species which can either condense with each other or react with nucleophilic function, such as the amino groups of chitosan. Although, the mechanism of the non-enzymatic reaction step is still poorly understood, experimental evidence appears to support the hypothesis that quinones can undergo (at least) two different types of reaction with amines to yield either Schiff-bases or Michael-type adducts, as well as oligomer-forming reactions with other quinones can be experimentally demonstrated (Kumar, Bristow, Smith, & Payne, 2000; Kumar, Smith, & Payne, 1999). By this way, chitosan can be enzymatically modified to create derivatives with a range of functional properties.

In general, functional properties are obtained by the association of this amino-polysaccharide with other biomacromolecules, as proteins (Babiker, 2002) or, more often, by the grafting of smaller molecules. For instance, Yamada et al. (2000) have successfully investigated the tyrosinase-catalyzed oxidation of 3,4-dihydroxyphenethylamine (dopamine) in the solutions of chitosan, producing gels with enhanced water-resistant adhesive properties. Similarly, Kumar et al. (1999) reported an improvement of the chitosan solubility, obtained by the tyrosinase-catalyzed grafting of chlorogenic acid onto the chitosan. In an other work, these authors showed that the reaction of chitosan with *p*-cresol, and using tyrosinase as biocatalyst, led to significantly enhance the ability of the chitosan to form high viscous solutions which can be converted into biodegradable gels (Kumar et al., 2000). With the same enzyme, Chen et al. (2000) studied the oxidation of the hexyloxyphenol and showed that the quinones bound covalently to the free amino groups of chitosan dissolved in the reaction medium. Recently, Sousa et al. (2009) showed the enhancement of antimicrobial and antioxidant properties of functionalized chitosan by the tyrosinase-catalyzed oxidation of flavonoids.

The purpose of this work is to study the mechanism of enzymatic grafting of phenolic compounds onto chitosan and to obtain, by simple and eco-friendly procedure, chitosan derivatives with improved or new functional properties. For this, chitosan solid particles were directly suspended in an aqueous medium. The ferulic acid (FA) and its ester (ethyl ferulate, FE) were chosen as phenolic substrates with and without free carboxylic groups. The *Myceliophthora thermophyla* laccase was utilized as biocatalyst which is able to oxidize *ortho*- and *para*-diphenols, methoxy-phenols, aromatic amines and monophenols (Karamyshev, Shleev, Koroleva, Yaropolov, & Sakharov, 2003), as ferulic acid and it is active in aqueous medium, at room temperature and neutral pH (Mayer & Staples, 2002; Minussi, Pastore, & Duran, 2002).

2. Materials and methods

2.1. Materials

Ferulic acid (FA) and ethyl ferulate (EF) (purity about 99%) were purchased from Sigma–Aldrich (France). HCl 37% and ferric chloride ($FeCl_3{\cdot}6H_2O$) were obtained from Merk (Germany), acetic acid and potassium ferricyanide [$K_3Fe(CN)_6$] were purchased from Prolabo (France). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), tetrafluoroacetic acid (TFA) and Chitosan HMW (high molecular weight, M_w 310–375 kDa) were purchased from Sigma–Aldrich (France); syringaldazine, trichloroacetic acid (TCA) from Fluka (France). Acetone, methanol, ethanol and acetonitrile were obtained from Carlo Erba (Milwaukee, WI, USA).

An industrial laccase named Suberase® (Novozymes, Bargsvaerdt, Denmark) was used in this study for the modification of chitosan particles. The Suberase® is a fungal laccase from *Myceliophthora thermophyla* produced by submerged fermentation of modified *Aspergillus orizae* strain. Laccase was partially purified by the ultrafiltration device with a cell Amicon-8200 (20 ml) equipped with a membrane of cellulose acetate (cut off 10 kDa) (SARTORIUS - 14539-63-G) under nitrogen.

2.2. Purification and characterization of the chitosan

To remove insoluble impurities from the chitosan and to obtain a homogeneous chitosan, a solution (1%, w/v) was prepared by dissolving chitosan powder in acetic acid (1%, v/v) and stirred overnight at 25 °C then filtered (0.22 μm) under vacuum. The filtrate was neutralized with a solution of NaOH (1 N) at pH 8. The obtained precipitate was thoroughly washed with distilled water then dried by lyophilisation. Later on, the dried chitosan was milled, mixed, packed in plastic bags and stored at 4 °C in cold room until needed.

The acetylation degree of chitosan (DA %) was determined by UV spectrophotometric method (Liu, Wei, Yao, & Jiang, 2006) and infrared spectroscopy method (Brugnerotto et al., 2001). The particle size average of chitosan was determined using the static light scattering (Mastersizer S, Malvern Instruments Ltd., Malvern, UK) with a 5 mW He–Ne laser operating at a wavelength of 632.8 nm with a 300RF lens. All measurements were carried out at least in triplicate.

The molar mass average of chitosan monomer was measured using Eq. (1):

$$M = M_d \times DD + M_a \times DA \quad (1)$$

where M_d is the molar mass average of deacetylated monomer (161 g mol^{-1}), M_a is the molar mass average of acetylated monomer (203 g mol^{-1}), DD the deacetylation degree and DA the acetylation degree.

The concentration of free amino groups from chitosan was measured using Eq. (2):

$$[-NH_2]\text{mol L}^{-1} = \frac{Cp \times DD}{100\,M}$$

where M is the molar mass average of chitosan monomer and Cp the chitosan rate in KBr (1% w/w) corresponding to infrared spectroscopy method.

2.3. Enzymatic oxidation of FA, EF and their grafting onto the chitosan

To evaluate the ability of chitosan particles to graft the products from laccase-catalyzed oxidation of FA or EF in an aqueous medium, the reactions were carried out at 30 °C in a magnetic stirred reactor under atmospheric conditions. In these experimental conditions, the dissolved oxygen was not limited for the reaction. Two reactions, with (I) and without (II) chitosan, were performed: for the reaction (I) 5 ml of methanol solution of 50 mM FA or EF, 45 ml of phosphate buffer (50 mM, pH 7.5) and 1 g of chitosan particles were mixed in the beaker. For the reaction (II) chitosan was not added, but the reaction medium was the same. The reactions were started by adding 0.13 ml of Suberase® (13.5 U/ml of laccase). Additionally, a control without laccase (III) and another control without substrate (FA or EF) (IV) were performed in the same conditions.

The reaction kinetic was monitored by High Performance Liquid Chromatography (HPLC) using the method described by Mustafa, Muniglia, Rovel, and Girardin (2005). In order to avoid solid chitosan particles, samples were carefully filtered with Ministar-RC membranes (Sartorius, porosity 0.2 μm) before analysis. Each analysis was made in triplicate.

The enzymatic reaction was stopped after 4 h, by filtering with Ministar-RC membranes (Sartorius, porosity 0.2 μm) the reaction

medium under vacuum. Later on, the chitosan recovered was extensively washed with an abundant amount of phosphate buffer pH 7.5, methanol, ethanol and then acetone to remove all traces of substrates and enzymatic proteins on the chitosan derivatives. Besides, this chitosan was chemically treated by HCl/ethanol (50:50) and by KOH/ethanol (50:50) at room temperature to verify the color stability.

2.4. Color measurement of the chitosan and its derivatives

The color of chitosan and its derivatives particles samples was assessed with equal little amounts (1 g) using colorimeter (model 200) (Montreuil, France) employing the parameters L^*(luminance) a^* (redness) and b^* (yellowness) defined in the CIELab system (Lathasree, Rao, Sivasankar, Sadasivam, & Rengaraj, 2004). The parameters a^*, b^* and L^* were measured directly in triplicate. Color difference (ΔE) was the magnitude of the resultant vector of three component differences: lightness difference (ΔL); red–green chromaticity difference (Δa); and yellow–blue chromaticity difference (Δb). Color differences were calculated using the following Eq. (3):

$$\Delta E = \sqrt{(\Delta L^2 + \Delta a^2 + \Delta b^2)} \quad (3)$$

where $\Delta a = a_i - a_0$, $\Delta b = b_i - - b_0$ and $\Delta L = L_i - L_0$. The index i, indicates the values observed for chitosan derivatives and index 0, indicates the values observed for chitosan (reference).

2.5. Structural characterization of the chitosan and its derivatives

All analyses were performed on the chitosan and its derivatives particles to confirm the products grafting of laccase-catalyzed oxidation of FA or EF onto the chitosan derivatives particles. For UV/visible spectra using spectrophotometer (Shimadzu UV-1605) scanning 240–600 nm, chitosan samples were dissolved at 0.5% (w/v) in aqueous acetic acid (1%) at pH 3 and then the samples were carefully filtered with Ministar-RC membranes (Sartorius, porosity 0.2 μm). For Fourier-transform infrared spectroscopy (FT-IR) analysis using a Tensor 27 (Bruker, Germany) with 128 scans, 10 kHz scanning and Spectra of 0–4000 cm^{-1}, samples were previously kept sheltered out from light and dried for 24 h at room temperature in a dessicator. The dried chitosan samples were ground and formed into a KBr pellets containing 1% (w/w) of chitosan before analysis. NMR experiments were performed on a Bruker AVIII-400 spectrometer operating at a ^{13}C frequency of 100.62 MHz and equipped with a double resonance H/X CP-MAS 4 mm probe. The MAS rate was fixed at 9000 Hz and each experiment was recorded at ambient temperature (293 ± 1 K). The cross polarization pulse sequence used a 3.7 μs 90° proton pulse, a contact time of 1 ms at 74 kHz and a 6 s recycle delay for an acquisition time of 34 ms during which a dipolar decoupling of 74 kHz was applied. A typical number of 8192 scans were acquired for each spectrum. Chemical shifts were calibrated by using the peak at 176.03 ppm of glycine as an external standard.

2.6. Estimation of the grafting efficiency

2.6.1. Measurement of the amino groups concentration

To estimate the grafting degree of phenolic compounds onto the chitosan, the concentration of the chitosan free amino groups was measured using Eq. (2) according to the infrared spectroscopy method (Brugnerotto et al., 2001).

The total acylation degree DA_{ct} of chitosan derivatives is the sum of the acetylation degree DA of chitosan and the acylation degree DA_c of acylated part of chitosan derivatives according to Eq. (4):

$$DA_{ct}\,ofchitosanderivatives = DAofchitosan + DA_c\,ofacylatedpart \quad (4)$$

where DA_c of acylated part is determined by the difference between DA of chitosan and chitosan derivatives by infrared spectroscopy method by the following Eq. (5):

$$DA_c = DAofchitosan - DAofchitosanderivatives \quad (5)$$

Additionally, the molar mass average of chitosan derivatives monomer (M_m) is the sum of the molar mass average of chitosan monomer ($M_d \times DD + M_a \times DA$) and of the molar mass average of acylated part ($M_c \times DA_c$) following Eq. (6):

$$M_m = M_d \times DD + M_a \times DA + M_c \times DA_c \quad (6)$$

where M_d is the molar mass average of chitosan deacetylated monomer (161 g mol^{-1}) and M_a is the molar mass average of chitosan acetylated monomer (203 g mol^{-1}).

In this work, it was supported that the products of laccase-catalyzed oxidation of FA or EF grafted onto the chitosan were of dimer type. Thus, the molar mass average of AF-chitosan derivative acylated dimer M_c is: $M_a + M_{AF} = 161 + 339 = 500$ g mol^{-1} while for M_c of FE-chitosan derivative acylated dimer is: $M_a + M_{FE} = 161 + 442 = 604$ g mol^{-1} where M_{AF} is molar mass of colored ferulic acid dimer (Mustafa et al., 2005) and M_{FE} is molar mass of colorless ethyl ferulate dimer (Ralph, Quideau, Grabber, & Hatfield, 1994).

2.6.2. Measurement of the electrophoretic mobility

Electro-kinetic property such as electrophoretic mobility was measured to determine the grafting efficiency. For measurement of electrophoretic mobility (μm cm/V s), chitosan samples were dissolved to reach a concentration of 2 mg/ml in aqueous acetic acid (1% v/v) at pH 3 and then carefully filtered with Ministar-RC membranes (Sartorius, porosity 0.2 μm) before analysis. All measurements of electrophoretic mobility of these solutions were performed at 25 ± 0.1 °C using Malvern Zetasizer Nano ZS (Malvern Instrument, England) equipped with a green laser 532 nm (Type: frequency doubled DPSS). Each measurement was repeated three times and the electrophoretic mobility average was recorded.

2.7. In vitro antioxidant properties of the chitosan and its derivatives

2.7.1. DPPH free radical scavenging activity

Each chitosan sample (0–1.5 mg/ml) in 1% (v/v) aqueous acetic acid (200 μl) was mixed with 1800 μl of methanolic solution containing DPPH• radicals resulting in a final concentration of 6×10^{-5} M DPPH•. The mixture was shaken vigorously for 15 s and then left to stand in the dark at room temperature for 30 min. The absorbance (Abs) of the resulting solution was measured at 517 nm using a UV–visible spectrophotometer (Shimadzu UV-1605) against a blank without chitosan according to the method of Shimada, Fujikawa, Yahara, and Nakamura (1992). The scavenging ability was calculated by the following Eq. (7):

$$DPPH^{\bullet}Scavengingability(\%) = (1 - Ab_{sample}/Abs_{control}) \times 100 \quad (7)$$

The EC_{50} value, which expressed the antioxidant concentration to reduce the radicals by 50%, was a good indicator to quantify the antioxidant capacity.

Each value represents the average ± standard deviation of three independent experiments.

2.7.2. ABTS radical cation decolorization assay

The spectrophotometric analysis of $ABTS^{\bullet+}$ radical scavenging activity was determined according to the method of Re et al. (1999). Chitosan (50–1000 μg/ml in 1% (v/v) aqueous acetic acid solution) was mixed with 1 ml of $ABTS^{\bullet+}$ solution which was produced by the reaction between 7 mM ABTS and 2.45 mM potassium persulfate.

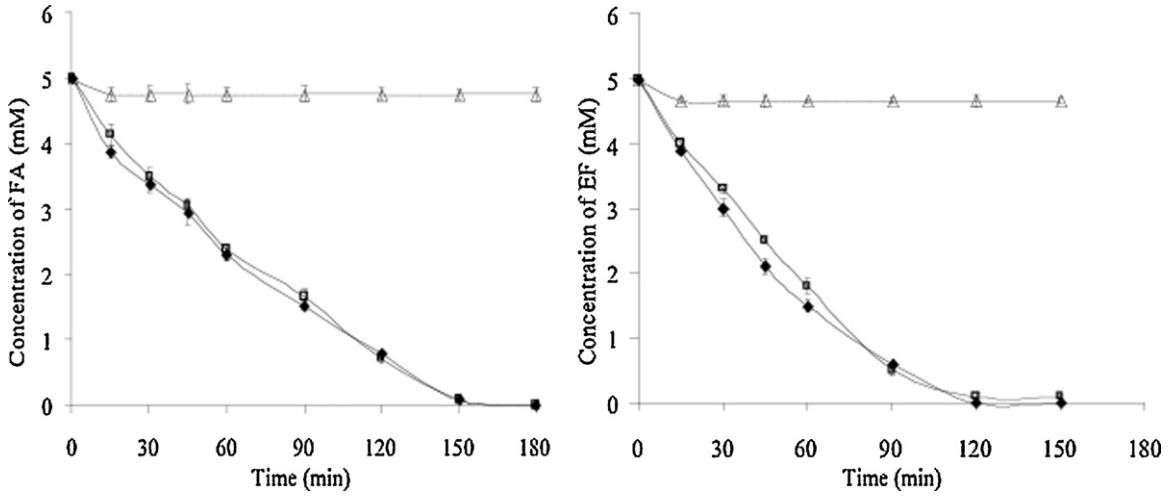

Fig. 1. FA or EF concentration in phosphate buffer (50 mM, pH 7.5) containing FA or EF + chitosan + laccase (□), FA or EF + laccase without chitosan (♦), FA or EF + chitosan without laccase (control) (Δ) at 30 °C. Results from triplicate experiments.

After 30 min in the dark, the absorbance of $ABTS^{\bullet+}$ was measured at 734 nm and the scavenging capability was calculated using Eq. (7).

In this study, $ABTS^{\bullet+}$ radical scavenging activity was determined by the calculation of EC_{50} value, which expresses the antioxidant concentration to reduce the $ABTS^{\bullet+}$ radicals by 50%. Values were the average ± standard deviation of three independent experiments.

2.7.3. Measurement of reducing power

The reducing power was determined according to the method of Oyaizu (1986). Chitosan (0–5 mg/ml in 1% (v/v) aqueous acetic acid solution) was added to 0.2 M sodium phosphate buffer (pH 6.6) and 1% (w/v) solution of potassium ferricyanide. After incubation at 50 °C for 20 min, 10% (w/v) trichloroacetic acid solution was added. Following this, the mixture was centrifuged at 10,000 × *g* for 10 min and the supernatant was then combined with distilled water and 0.1% (w/v) solution of ferric chloride. Results were expressed as content of chitosan sample (mg/ml) in function of absorbance at 700 nm. Values were the average ± standard deviation of three independent experiments.

3. Results and discussion

3.1. Purification and characterization of the chitosan

The characterization of the purified chitosan gave a deacetylation degree of 83 ± 1%, a particule size average of 220 ± 2 μm, a molar mass average of monomer of 159.8 ± 7.0 g mol^{-1} and a concentration of free amino groups of 0.050 ± 0.002 mol L^{-1}.

3.2. Enzymatic oxidation of FA, EF and their grafting onto the chitosan

The laccase-catalyzed oxidation of FA or EF in the presence or absence of chitosan was studied and similar results were obtained as shown in Fig. 1. These results suggested that the presence of chitosan did not significantly interfere with the oxidative rate of FA or EF by laccase. As laccase remained active after 4 h of reaction in comparison with the reaction in the absence of chitosan (data not shown), it appeared that chitosan could protect the enzyme from inhibition during the oxidation of FA or EF. These authors demonstrated that the stability of laccase is presumably due to the rapid adsorption of the reactive quinones onto chitosan. In contrast, these results appeared to be different from those reported by other authors (Sun, 1992; Wada, Ichikawa, & Tatsumi, 1993) who showed that the rate of reaction of several substrates including phenol were accelerated in the presence of chitosan. Moreover, as shown in Fig. 1, if the laccase was not added to the system, the initial concentration of FA or EF decreased for about 7.5–8% for 1 g of chitosan addition and this percentage increased with an increasing quantity of chitosan. This result indicated that physicochemical interactions between FA and EF and the chitosan particles probably occured. This phenomenon is in good agreement with the results reported by other authors (Sousa et al., 2009) who found physicochemical interactions between chitosan particles and flavonoids in the presence of tyrosinase. Additionally, Murata, Nagaki, Kofuji, and Kishi (2010) reported that an electrostatic complex between chitosan and FA was formed during the preparation of chitosan-FA salt in a suspension of deionized water.

Additionally, it was measured that laccase was adsorbed physically onto the chitosan particles for about 11% at neutral pH. The adsorbed enzyme was not active in these conditions probably due to a reduced accessibility of the active site or the denaturation of the enzymatic structure as already reported by other authors (Berger et al., 2004; Carunchio, Crescenzi, Girelli, Messina, & Tarola, 2001; Delanoy, Li, & Yu, 2005).

Moreover, the FA-chitosan derivative color was gradually modified towards yellow–orange while the EF-chitosan derivative remained colorless. Thus, it indicated that chitosan stabilized the colored and the colorless products formed in the initial steps of the substrate oxidation according to the results of Sousa et al. (2009) who obtained a colored chitosan by grafting of products from tyrosinase-catalyzed oxidation of flavonoids.

After 4 h, the chitosan derivatives was recovered and washed with different solvents. Besides, FA-colored chitosan derivative has been chemically treated by HCl/ethanol and by KOH/ethanol at room temperature. In both cases, colored chitosan preserved its visual color after the neutralization to pH 7.5. These observations suggested that the links between FA or EF products and the chitosan were of covalent nature. To confirm this hypothesis, UV/visible, FT-IR and ^{13}C NMR analyses of the chitosan derivatives were performed.

3.3. Color measurement of the chitosan and its derivatives

The values obtained for color parameters (a^*, b^* and L^*) and ΔE values of chitosan particles were shown in Table 1. The values of color parameters (a^*, b^* and L^*) of AF-chitosan derivative were

Table 1
Color parameters (a^*, b^* and L^*) and color difference (ΔE) of chitosan and its derivatives particles.

	L^* (Lightness)	a^* (Redness)	b^* (Yellowness)	ΔE (Color difference)
Chitosan	89.20 ± 0.10	0.10 ± 0.04	7.70 ± 0.14	0
FA-chitosan derivative	60.50 ± 0.30	21.50 ± 0.10	33.90 ± 0.41	44.40 ± 1.60
EF-chitosan derivative	85.20 ± 0.15	0.30 ± 0.02	8.20 ± 0.12	4.70 ± 0.20

Each value is expressed as mean ± standard deviation ($n = 3$).

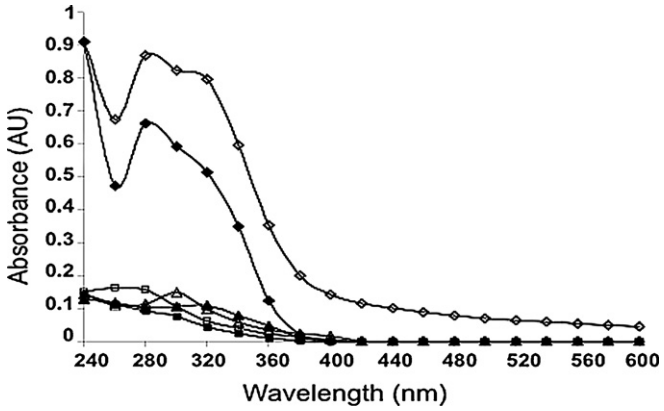

Fig. 2. UV/visible spectra for chitosan (■), chitosan control with laccase (□), chitosan control with FA (▲), chitosan control with EF (△), FA-chitosan derivative (◊), EF-chitosan derivative (♦), and after washing with different solvents, chitosan samples were dissolved at 0.5% (w/v) in aqueous acetic acid 1% (pH 3) and then filtrated prior to measuring the spectra.

significantly different ($\Delta E = 44.36$) when compared with those of chitosan. Indeed, with the products grafting of laccase-catalyzed oxidation of FA onto the chitosan, redness and yellowness increased and lightness decreased leading to a colored chitosan derivative (yellow–orange). On the other hand, the color parameters of chitosan and EF-chitosan derivative were very similar and color difference ΔE was not significant as shown in Table 1. Therefore, EF-chitosan derivative remained colorless.

3.4. Structural characterization of the chitosan and its derivatives

The aim of these chemical analyses was to provide chemical evidence that the products of laccase-catalyzed oxidation of FA or EF reacted with chitosan amino groups and then to identify specific linkage.

For the chitosan derivatives solution, significant changes in the UV/Vis spectra were observed (Fig. 2). A large increase of the absorbance between 300 and 350 nm was observed probably due to a reaction between the products of laccase-catalyzed oxidation and free amino groups of chitosan (Muzzarelli, Littarru, Muzzarelli, & Tosi, 2003; Nithianandam & Erhan, 1998; Ravi Kumar, 2000). Moreover, the large increase in the absorbance at 280 nm was probably caused by physically partial adsorption of laccase onto chitosan. Additionally, as presented in Fig. 2, the FA-chitosan derivative absorbed in the visible-region (colored chitosan) while the EF-chitosan derivative did not show any absorbance in this region (colorless chitosan). Thus, these results suggested that products synthesized by laccase-catalyzed oxidation of FA or EF could be covalently grafted onto the chitosan derivatives.

The second approach to characterize the products grafting of laccase-catalyzed oxidation of FA or EF consisted in the measurement of FT-IR spectra of the chitosan derivatives discussed above. Fig. 3 showed that when compared with the chitosan, the chitosan derivatives presented an absorption decrease at 1320 and 1380 cm^{-1} (attributed to the NH-bending of the glucosamine unit)

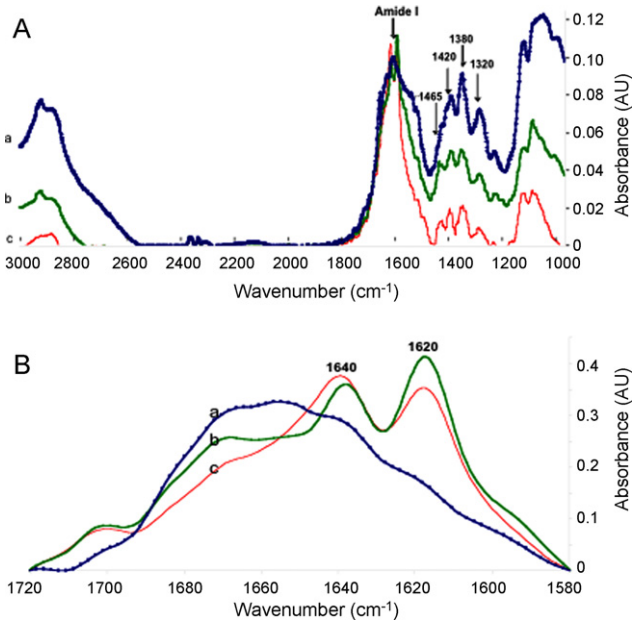

Fig. 3. FT-IR spectra (**A**) and amide I band (**B**) of chitosan (a), EF-chitosan derivative (b) and FA-chitosan derivative (c).

(Sousa et al., 2009), and at 1420 cm^{-1} (the symmetric $-NH_3^+$ bending region) (Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982) which may be associated with protonated glucosamine residues of chitosan. The decrease of these bands indicated a loss of NH_3^+ groups that was consistent with a covalent reaction between the products of laccase-catalyzed oxidation of FA or EF and the chitosan amino groups. In addition, the formation of a new band at 1465 cm^{-1} can be attributed to a phenyl group of phenolic compounds (Muzzarelli & Ilari, 1994). Thus, the changes observed at the 1000–1500 cm^{-1} region can be attributed to the aromatic C–O and aliphatic C–O bond stretching of FA or EF according to results reported by Soto, Freer, and Baeza (2005) for chitosan glyoxylate. Moreover, in the FT-IR spectra of chitosan derivatives, new bands appeared at 1620 and 1640 cm^{-1} corresponding to the C=N vibrations that was characteristic of imines and C=C stretching vibrations due to Schiff base formed between the amino groups NH_3^+ of chitosan pyranose cycle with the phenolic moieties. This result was in good agreement to results obtained by Jin, Wang, and Bai (2009) who confirmed that strong bands at 1620 and 1640 cm^{-1} may correspond to Schiff base bond between amino groups of chitosan with citral. These modifications in the spectra suggested that chitosan amino groups were subjected to covalent modifications of Schiff base type by the products of laccase-catalyzed oxidation of FA or EF.

Further characterization of chitosan derivatives was performed using Solid-state ^{13}C nuclear magnetic resonance (^{13}C NMR). The ^{13}C NMR spectrum of chitosan and chitosan derivatives were shown in Fig. 4(A). Characteristic signals from oligosaccharides were observed between 45 and 120 ppm with additional peaks corresponding to aliphatic carbons around 23 ppm and carbonyl

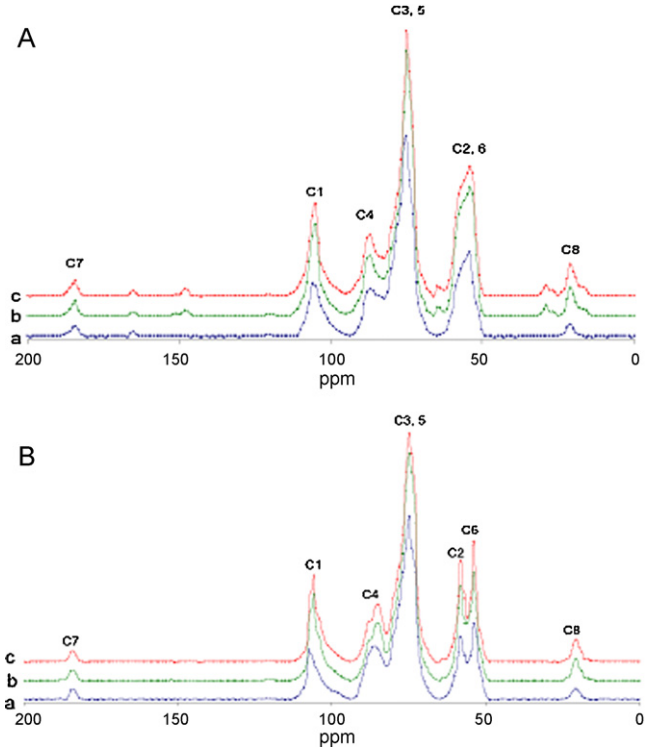

Fig. 4. (**A**) ^{13}C NMR spectrum of chitosan (a), FA-chitosan derivative (b) and EF-chitosan derivative (c). (**B**) ^{13}C NMR spectrum of chitosan hydration (a), FA-chitosan derivative hydration (b) and EF-chitosan derivative hydration (c).

carbons in the 170–180 ppm region. Signal centered at 164.6 ppm was tentatively assigned to a rotation band.

The large signals centered at 58, 74.6, 82.8 and 104.8 ppm were assigned to C-2 and C-6, C-3 and C-5, C-4 and C-1 carbons respectively according to previous studies (Huang, Mendis, Rajapakse, & Kim, 2006; Saitô, Tabeta, & Ogawa, 1987). The methyl and carbonyl functions of acetylglucosamine were represented by C-7 and C-8 carbons around 174 ppm (C=O) and 23 ppm (CH_3) respectively. Additional peaks were observed in the spectrum of FA and EF chitosan derivatives, especially the signal at 66.2 ppm may be attributed to the CH_2 of ethyl group, signals at 28 and 30.62 ppm were characteristic of unshielded CH_2 groups or OCH_3 while the signal at 19.56 ppm may correspond to a methyl of EF. These additional peaks clearly indicated that the products of laccase-catalyzed oxidation of FA or EF were grafted onto chitosan. In order to improve the spectral resolution and to distinguish the signals of C-2 and C-6, the hydration of the chitosan samples was performed using a saturated salt solution of NaCl. In general, conditioning of chitosan samples in this condition permitted to hydrate samples with 15% of water (w/w) (Gocho, Shimizu, Tanioka, Chou, & Nakajima, 2000). Fig. 4 shows the resulting ^{13}C NMR spectrum of chitosan and its derivatives. Most notable are the duplication of the C-2 signal around 58 ppm and the deformation of C-4 signal. The multiplicity of C-4 observed in Fig. 4 was attributed to different conformations of the glucosidic bond between the anomeric C-1 and C-4 carbon and/or the presence of different polymorphs of chitosan. The duplication of C-2 signal could be attributed to the presence of a new conformation of C-2 due to the phenolic moieties grafting onto the chitosan derivatives. These results suggested that the products of laccase-catalyzed oxidation were grafted onto the chitosan derivatives at position C-2 that was consistent with results reported by other authors (Sampaio, Taddei, Monti, Buchert, & Freddi, 2005).

3.5. Estimation of the grafting efficiency

3.5.1. Measurement of the amino groups concentration

The values DA_c, DA_m, M_m and percentage of bound NH_2 of chitosan derivatives increased with the products grafting of laccase-catalyzed oxidation of FA or EF onto the chitosan derivatives in comparison with the chitosan were shown in Table 2. Furthermore, these values of FA-chitosan derivative were higher than EF-chitosan derivative. These results indicated that the quantity of FA-products grafted onto the chitosan was higher than EF-products.

3.5.2. Measurement of the electrophoretic mobility

Electrophoretic mobility (EM) measurements were performed at pH 3 at concentration 2 mg/ml in aqueous acetic acid 1% (v/v) in triplicates. The electrophoretic mobility value of chitosan was determined as 6.97 ± 0.07 μm cm/V s as chitosan is positively charged due to free amino groups. This value was found to decrease with the products grafting of laccase-catalyzed oxidation of FA or EF onto the chitosan (5.41 ± 0.10, 6.24 ± 0.12 μm cm/V s) respectively. Additionally, the electrophoretic mobility decrease for FA-chitosan derivative solution was higher than for EF-chitosan derivative solution when compared with the electrophoretic mobility of chitosan. These results suggested that the quantity of FA-products grafted onto the chitosan was higher than EF-products.

3.6. In vitro antioxidant properties of the chitosan and its derivatives

3.6.1. DPPH free radical scavenging activity

The comparative EC_{50} values of chitosan and its derivatives were shown in Table 3. In the case of chitosan, no reduction of DPPH• was observed as reported by other authors (Pasanphan, Buettner, & Chirachanchai, 2010; Wu, Tseng, & Juang, 2001). This may be partly due to the inhibition of radical scavenging because of inter- and intramolecular hydrogen bonding. Although, the EC_{50} values in DPPH• scavenging of FA-chitosan derivative and EF-chitosan derivative were determined as 0.52 ± 0.04 mg/ml and 1.50 ± 0.04 mg/ml, respectively. The scavenging activity of FA-chitosan derivative was three times higher than EF-chitosan derivative which can be attributed either to high antioxidant properties of pure FA (Nenadis, Zhang, & Tsimidou, 2003) or to high quantity of the products of laccase-catalyzed oxidation of FA grafted onto chitosan when compared with pure EF. Thus, the introduction of an H-atom donating group, which was produced from laccase-catalyzed oxidation of FA or EF onto chitosan, appeared to be a good strategy to develop a chitosan derivative with increased antioxidant capacity leading to an antioxidant polymer.

3.6.2. ABTS radical cation decolorization assay

The EC_{50} values in scavenging the $ABTS^{\bullet+}$ of the chitosan and its derivatives were also determined (Table 3). In the case of the chitosan, no reduction of $ABTS^{\bullet+}$ was observed and this may be partly due to the inhibition of radical scavenging because of inter- and intra-molecular hydrogen links. The EC_{50} values in scavenging the $ABTS^{\bullet+}$ of FA-chitosan derivative and EF-chitosan derivative were 0.20 ± 0.02 mg/ml and 0.66 ± 0.05 mg/ml, respectively. The scavenging activity of FA-chitosan derivative was nearly three times higher than EF-chitosan derivative. Thus, an enhancement of the antioxidant activity in the chitosan derivatives with the products of laccase-catalyzed oxidation of FA or EF was demonstrated.

3.6.3. Measurement of reducing power

In agreement to previous studies (Kanatt, Chander, & Sharma, 2008), Fig. 5 shows that the chitosan presented almost negligible

Table 2
Values of the acetylation degree (DA), the deacetylation degree (DD) the acylation degree (DA_c) of acylated part, the total acylation degree (DA_{ct}), the molar mass average of chitosane monomer (M_m) and the concentration of free amino groups [$-NH_2$] of chitosan and its derivatives regarding to this work.

	A_{1320}/A_{1420}	DA%	DA_c%	DA_{ct}	DD%	M_m	[$-NH_2$] mol L^{-1}
Chitosan	0.95	18.0	0	18.0	82.0	168.6	0.049
EF-chitosan derivative	0.75	11.8	6.2	24.2	75.8	208.6	0.036
FA-chitosan derivative	0.55	5.6	12.4	30.4	69.6	235.7	0.030

Table 3
EC_{50} values of chitosan and its derivatives in antioxidant properties.

	EC_{50} (mg/ml)		
	Chitosan	FA-chitosan derivative	EF-chitosan derivative
Scavenging ability on DPPH radicals	–	0.52 ± 0.04	1.50 ± 0.04
Scavenging ability on ABTS radicals	–	0.20 ± 0.02	0.66 ± 0.05

EC_{50} value corresponds to the effective concentration necessary at which the antioxidant activity was 50%, EC_{50} values were obtained by interpolation from linear regression analysis. Each value is expressed as mean ± standard deviation ($n = 3$).

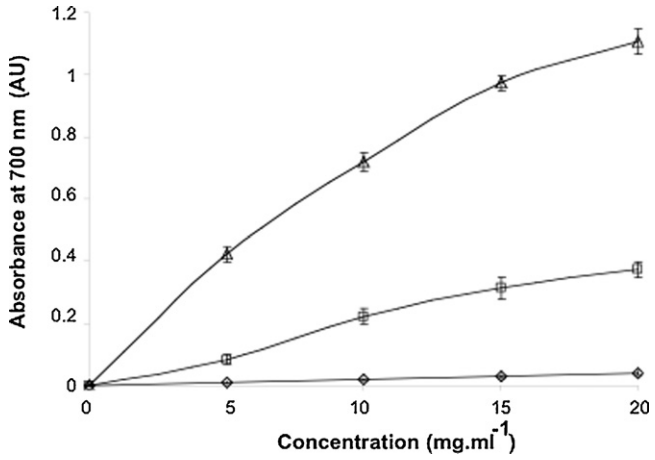

Fig. 5. Reducing activity of FA-chitosan derivative (△), EF-chitosan derivative (□) and chitosan (◊). Each value is expressed as mean ± standard deviation ($n = 3$).

reducing power while it increased with increasing FA or EF-chitosan derivative concentration. Moreover the reducing power of FA-chitosan derivative was higher than EF-chitosan derivative. In general, the reducing properties are associated with the presence of reductones which exhibit antioxidant action by breaking the free radical chain by donating a hydrogen atom (Duh, 1998).

With the grafting of the products of laccase-catalyzed oxidation of FA or EF onto chitosan particles, the antioxidant properties were enhanced when compared with chitosan particles. It was observed that these enhancements were dependent on the antioxidant assay of the phenols used as well as on the quantity of the grafted phenols. Thus, the results of DPPH, ABTS radical scavenging activities and those of reducing power suggested that chitosan derivatives could be considered as an efficient antioxidant polymer.

4. Conclusion

In this work, the functionalization of chitosan particles by the oxidative products of FA or EF catalyzed by the *Myceliophtora thermophyla* laccase was investigated. This enzymatic reaction was carried out under mild operational conditions (at 30 °C and pH 7.5), with the solid chitosan particles suspended in the reaction medium, without harsh acidic solubilization and without organic solvent.

In summary, the laccase from *Myceliophtora thermophyla* could oxidize FA and EF to reactive radicals and the oxidative rate of EF appeared higher than that of FA. The presence of chitosan in the reaction medium did not influence the oxidative rate of FA or EF and it could protect the enzyme from inhibition of oxidative products during the oxidation. Additionally, the enzymatic functionalization led to colored and colorless chitosan derivatives with improved functional properties.

This finding hinted that the oxidative products of FA or EF were covalently bound onto the chitosan free NH_2 groups at C-2 region of the chitosan particles by Schiff reaction and the grafting level of AF-oxidation products onto the chitosan was higher than that of EF-oxidation products. Additionally, the chitosan derivatives presented improved antioxidant properties especially for FA-chitosan derivative when compared with chitosan.

These results demonstrated the interests for using renewable resources and biochemical processing to create functional polymers. These innovative molecules present higher value-added properties and could be a model for applications in important biotechnological domains such as active food-packaging and antioxidant additives for foods as well as for cosmetics.

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