Carbohydrate Polymers 87 (2012) 2388–2398

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Laccase-mediated functionalization of chitosan by caffeic and gallic acids for modulating antioxidant and antimicrobial properties

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ARTICLE INFO

Article history:
Received 3 August 2011
Received in revised form 29 October 2011
Accepted 3 November 2011
Available online 12 November 2011

Keywords:
Chitosan
Phenolic acids
Laccase
Functionalization
Antimicrobial and antioxidant activity

ABSTRACT

A new strategy for the functionalization of chitosan with caffeic acid (CA) or gallic acid (GA) using laccase from *Trametes versicolor* is presented for the first time, yielding a product with modulated antioxidant and antimicrobial properties. UV–vis spectroscopy coupled to HPLC-SEC analysis and cyclic voltammetry kinetic studies showed that laccase catalyzes the oxidation of phenolic acids to electrophilic *o*-quinones, which undergo new oligomer/polymer-forming structures originated by C–C coupling between the benzene rings and C–O–C coupling involving phenolic side-chains. Furthermore, pH tunable reactions/interactions of the laccases oxidized *o*-quinones with nucleophilic amino groups of chitosan were determined with FTIR and ^{1}H NMR spectroscopy's. The highest antioxidant activity was found to be for chitosan modified with phenolic acids at pH 4.5, exhibit also an increased activity against *Escherichia coli* and *Listeria monocytogenes* compared to untreated chitosan.

1. Introduction

The development of highly performable bio-functional materials by using bio-based polymers from renewable resources has gained considerable attention in last decade, as emphasized by numerous reviews on the topic (Muzzarelli et al., 2011; Ravi Kumar, 2000; Zhang et al., 2010). Chitosan has proved to be biodegradable, non-toxic, non-antigenic, and a biocompatible polysaccharide polymer with excellent antimicrobial properties (Muzzarelli & Muzzarelli, 2009). However, its poor antioxidant activity the lack of a H-atom donor to serve as a good chain-breaking antioxidant (Sun, Zhou, Xie, & Mao, 2007), especially for chitosan of medium and high-molecular weights, limits its application in many areas (Aranaz et al., 2009). For example wound healing materials and food packaging it is essential to provide, besides antimicrobial inhibition, also a reduction of reactive oxygen species, which are strongly implicated in the pathogenesis of e.g., wounds, causing injury to bio-molecules such as lipids, proteins, and nucleic acids, as well as the depletion of mitochondrial DNA from the human skin (Schroeder, Gremmel, Berneburg, & Krutmann, 2008), and/or e.g., retarding the degradation reactions of fats and pigments in food products (Murata, Nagaki, Kofuji, & Kishi, 2010).

Gallic (GA) and caffeic (CA) acids are well known natural phenolic antioxidants which have been already chemically applied to chitosan polymer in order to accelerate its antioxidant activity (Aytekin, Morimura, & Kida, 2011; Cho, Kim, Ahn, & Je, 2011; Pasanphan & Chirachanchai, 2008; Pasanphan, Buettner, & Chirachanchi, 2010; Shiu et al., 2010; Yu et al., 2011). Oxidative enzymes such as tyrosinase and peroxidase have also been shown as potential region- and stereo-selective catalysts for polymerization of phenolic substrates as well as for grafting of oxidized phenols onto the chitosan macromolecule (Fras-Zemljič, Kokol, & Čakara, 2011; Muzzarelli, Ilari, Xia, Pinotti, & Tomasetti, 1994; Sousa, Guebitz, & Kokol, 2009), being an attractive alternative to toxic, environmentally unfriendly, and non-specific chemical approaches. However, there are still no reports on using laccase as reaction catalyst in phenolic–chitosan modifications, although they are known to catalyze the oxidation of various phenol-like compounds, aromatic amines, and some inorganic compounds. Beside, laccases do not require any additional initiators as, e.g., H_2O_2, whilst its low pH optimum can be exploited for modifying chitosan that becomes soluble under pH of 6.1. However, since the relative oxidation rates of the phenolic compounds initiated by laccase are highly dependent to various reactions conditions, and since the antioxidant activities of phenols depend on the number and arrangement of the hydroxyl groups, and the extent of its structural conjugation, a precise analysis overview of its grafting chemistry and efficiency is required in order to control the properties of the final products.

Therefore, the aim of this study was to ascertain the influence of pH-depended laccase oxidation of structurally similar phenolic acids, CA and GA, and their reaction with chitosan with the purpose to produce the phenolic-functionalized chitosan films with different but synergistically active antioxidant and antimicrobial properties.

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doi:10.1016/j.carbpol.2011.11.006

2. Materials and methods

2.1. Materials

Medium molecular weight chitosan (M_w of 421.5 kDa, determined by HPLC/SEC) with a degree of deacetylation ~ 90, was purchased from Mahtani Chitosan Pvt. Ltd., India. Gallic acid (3,4,5-trihydroxybenzoic acid, GA), caffeic acid (3-(3,4-dihydroxyphenyl)-2-propenoic acid, CA), and laccase from *Trametes versicolor*, were obtained from Sigma–Aldrich, and used without further purification. All reactions were conducted in deionized water. Other reagents used were of analytical grade.

2.2. Laccase activity

The activity of the laccase was determined by monitoring the oxidation of ABTS at 436 nm ($\varepsilon_{436} = 2.9 \times 10^4\ M^{-1}\ cm^{-1}$) as reported by Niku-Paavola, Karhunen, Salola, and Raunio (1988). The assay reaction mixture contained a 200 μL enzyme solution, 100 μL of freshly prepared 1 mM ABTS, and a 700 μL 100 mM citrate buffer of pH 4.5. Enzyme activity was expressed in units, U, defined as μmol of ABTS oxidized per min. The pH and temperature activities profiles of the laccases were determined within the pH of 4.5–7.5 at room temperature, and at pH 4.5 within the 20–70 °C.

2.3. Polymerization of phenolic acids by laccase

CA and GA monomers were dissolved at 10 mM concentration in a 100 mL phosphate buffer (100 mM) of pH 6.5, and then reacted with 153 U of laccase for 24 h at 30 °C under constant stirring. The reaction mixtures were collected, centrifuged for 2 h at 11,000/min and washed off three times with water to eliminate non-reacted monomers. The retentate containing the oligomers/polymers was frozen at −20 °C and then lyophilized for 24 h using Mini-lyotrap LTE equipment. The oligomers/polymers were maintained at −20 °C until use.

2.4. Size exclusion chromatography

Size-exclusion chromatography (SEC) was used to evaluate the molecular weights (M_w) of the chitosan and synthesized phenolic oligomers/polymers. These analyses were performed using a High-Performance Liquid Chromatography Series 1200 (Agilent Technologies), using a Plaquagel-OH column. Retention was detected on the Refractive Index Detector (RID) and the Variable Wavelength Detector (VWD), with a detection interval of 190–400 nm equipped with a standard cell. A chitosan solution (addition of HCl) was diluted to a concentration of 1 g/L, filtered, and injected within the system having a 0.5 M $NaNO_3$ and 0.01 M NaH_2PO_4 mobile phase, with a flow-rate of 1 mL/min. Linear polyethylene standards were used to record the calibration curve when determining chitosan M_w. The phenolic oligomers/polymers were dissolved (4 days) in tetrahydrofuran (1 g/L) and eluted through a Zorbax PSM column. The same solvent was used during the mobile phase, with a flow-rate of 1 mL/min. Polystyrene standards with molecular weights between 1000 and 4000 g/mol were used for column calibration/estimation of the samples molecular weights. All HPLC/SEC data were evaluated using Chem Station computer software (Agilent Technologies).

2.5. Electrochemical experiments

Voltammetric experiments were performed using a PGSTAT101 Autolab controlled by Autolab NOVA software version 1.5. All the experiments were carried out in a 100 mL Metrohm cell with a triple-electrode configuration. The working electrode was a glassy carbon (GCE) with a surface diameter of 3 mm (Metrohm). The counter and reference electrodes were platinum (Metrohm) and Ag/AgCl (Metrohm) electrodes, respectively. The renewal of the glassy carbon surface was achieved by polishing with 1.0 and 0.3 μm alpha-alumina (Micropolish, Buehler) on a micro cloth-polishing pad (Buehler), followed by washing in an ultrasonic Selecta bath for 2 min. The phenolic compounds' (10 mM) oxidation by laccase was studied using 153 U of laccase in 100 mM phosphate buffer (pH 6.5) at a 50 mV/s scan rate, within a thermostated-cell (100 mL) at 30 °C, and the voltammetric curves were recorded every hour for 5 h, and then after 24 h.

2.6. Preparation of chitosan films

Chitosan was added to water, and a small amount of HCl was added incrementally to the solution in order to maintain the pH at around 3. After the undissolved material was centrifuged out, the chitosan solution was diluted to 1% *w*/*v*, and the pH was adjusted to 5.8–6.0 using NaOH. Chitosan films were prepared by adding 5 mL of a 1% *w*/*v* chitosan solution to 7 cm-diameter Petri dishes, and then oven-dried overnight at 45 °C. The dried films were neutralized by immersion in 1 M NaOH for 3 h, thoroughly washed with water and phosphate buffer (100 mM, pH 6.5), and stored in phosphate buffer at 4 °C.

2.7. Enzymatic functionalization of chitosan

In order to study the pH-dependent grafting efficacy of laccase-oxidized phenolic to chitosan, being insoluble under pH 6.1, two procedures were applied.

Heterogeneous grafting was conducted by incubating the chitosan films for 24 h in 100 mL buffer (100 mM citrate buffer, pH 5.5 or 100 mM phosphate buffer, pH 6.5), containing phenolic (10 mM) and laccase (153 U), under constant shaking at 30 °C. The control samples were treated in the same way but without adding the enzyme. After the reaction, the films were rinsed out extensively with ethanol and water.

Homogeneous grafting was performed using previously prepared chitosan (1% *w*/*v*), phenolic (10 mM), and laccase (153 U) solutions, each of them adjusted to a target value of pH 4.5. The chitosan solution was heated to 30 °C and an individual phenolic solution was added under constant stirring. After homogeneous distribution laccase solution was slowly added whilst stirring, and allowed to react for 24 h at 30 °C. The laccase chitosan conjugates were removed from the solution by centrifugation at 5000/min and 4 °C. The solutions were then cast onto 7 cm-diameter Petri dishes and oven-dried overnight at 45 °C. The dry films obtained were peeled off and stored at 4 °C until analysis.

2.8. ATR-FTIR analysis

The spectra of chitosan films, heterogeneously and homogeneously functionalized chitosan films with laccase-oxidized GA and CA, theirs monomers and laccase-synthesized oligomers/polymers were recorded using a Perkin-Elmer Spectrum One FTIR spectrometer with a Golden Gate ATR attachment and a diamond crystal. The absorbance measurements were carried out within the 650–4000 cm^{-1}, with 16 scans and a resolution of 4 cm^{-1}.

2.9. ^1H NMR analysis

^1H NMR spectra were acquired on a Varian Unity Inova 300 MHz spectrometer. The experiments were run at 25 °C (chitosan) or at 65 °C (heterogeneously functionalized chitosan), a temperature at which the solvent (CD_3COOD/D_2O) peaks do not interfere with any of the chitosans peaks.

2.10. Antioxidant activity

The antioxidant activity was determined by mixing 1 mg of the test sample and a 300 μL $ABTS^{\bullet+}$ free radical solution prepared by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulphate, stored in the dark at room temperature for 12 h. Before usage, the $ABTS^{\bullet+}$ solution was diluted with phosphate buffer saline (PBS) in order to reach an absorbance of 0.700 ± 0.025 at 734 nm. The inhibition of the $ABTS^{\bullet+}$ radical was monitored at 734 nm and 25 °C, and the percentage inhibitions of this radical were calculated at the ends of 10 and 30 min using Eq. (1), where $A_{control}$ is the initial concentration of the $ABTS^{\bullet+}$ and A_{sample} is the absorbance of the remaining concentration of $ABTS^{\bullet+}$ in the presence of the sample. All data are the averages of triplicate experiments.

$$\text{Inhibition or scavenging effect } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (1)$$

2.11. Antimicrobial activity

The antimicrobial properties of the treated samples were evaluated by the American Standard Test Method (ASTM) E2149-01 (Standard test method for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions) at accredited Institute of Public Health Maribor, Department of Microbiology. *Escherichia coli* (*E. coli*), *Salmonella enterica* (*S. enterica*) a gram-negative bacterium, *Listeria monocytogenes* (*L. monocytogenes*) a gram-positive bacterium and *Candida albicans* (*C. albicans*) fungus, were selected due to their resistance to common antimicrobial agents. The incubated test culture in a nutrient broth was diluted using a sterilized 0.3 mM phosphate buffer (KH_2PO_4; pH 6.8) to give a final concentration of $1.5–3.0 \times 10^5$ colony-forming units (CFU)/mL. This solution was used as a working microorganism suspension. The chitosans' films were cut into small pieces (1 cm × 1 cm) and transferred to a 250 mL Erlenmeyer flask containing 50 mL of the working suspension. All flasks were loosely capped, placed in the incubator, and shaken for 1 h at 37 °C and 120/min using a Wrist Action incubator shaker. The initial level of micro-organisms was determined in the following way: a sample (1 mL) was serially diluted and plated (inoculated) in nutrient agar. The inoculated plates were incubated at 37 °C for 24 h and the surviving cells were counted (B). The flasks were further shaken immediately at maximum speed for 1 h, after which the bacterial level (A) was determined in the same way as the initial level. The relative reduction in the number of micro-organisms (R) was calculated, by the following equation (Lim & Hudson, 2004):

$$\text{Reduction rate}: \quad R(\%) = \frac{B - A}{A} \times 100 \quad (2)$$

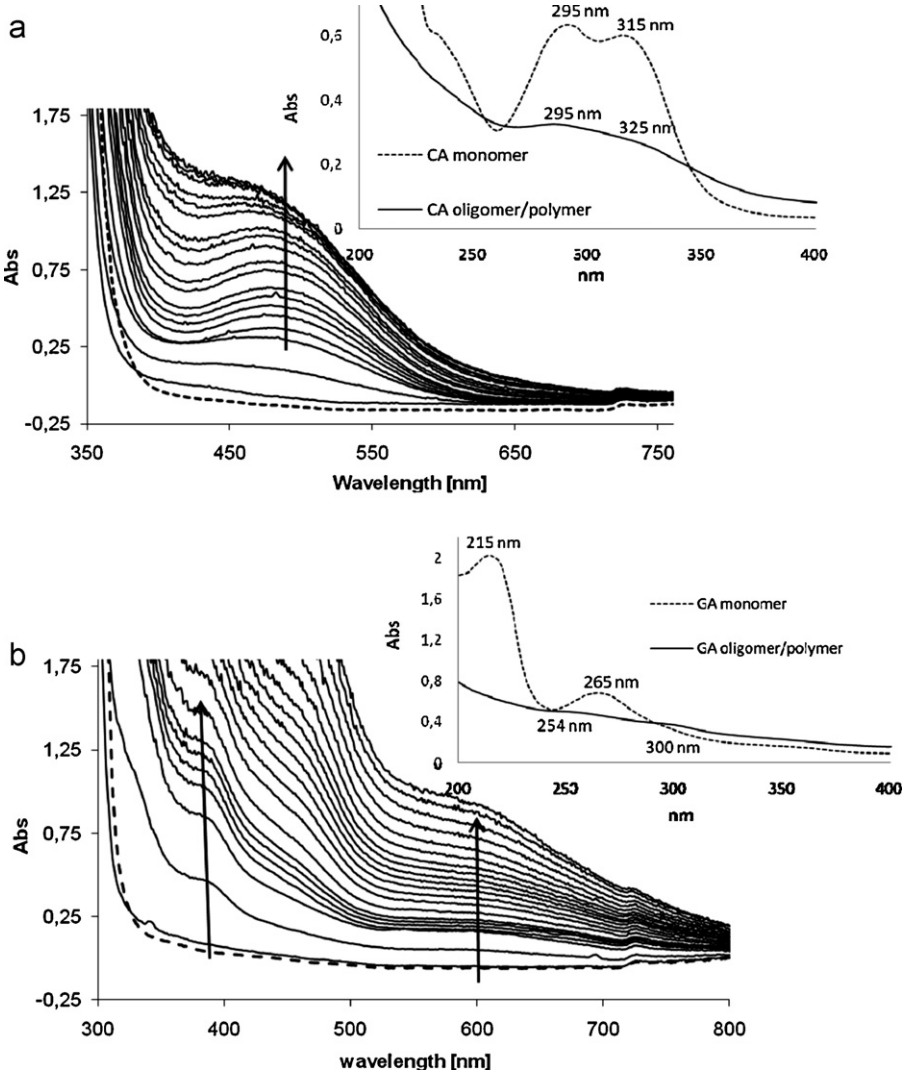

Fig. 1. UV–vis evidence of the (a) CA and (b) GA polymerization by laccase at pH 6.5. The arrows show the evolution of the bands.

3. Results and discussion

3.1. Laccase-catalyzed oxidation of phenolic acids in the absence and presence of chitosan

The laccase-catalyzed oxidations of CA and GA to reactive *o*-quinones, their polymerization and/or subsequent non-enzymatic reactions with the primary amino groups of chitosan were examined under different pH conditions using UV–vis spectroscopy and cyclic voltammetry.

As presented in Fig. 1 for both CA and GA, significant changes in the UV–vis spectra were observed during reactions with laccases at pH 6.5. Whilst a new broad-band between 450 and 550 nm appeared for CA, two new bands at 390 nm and 600 nm were observed for GA, which additionally increased over time. The spectra analysis of the control solutions, containing only CA or GA, showed no spectrum variation during the time, thus suggesting that no substantial auto-oxidation occurred at pH 6.5 over a 24 h period.

Two UV absorption bands were detected for both phenolic acids at pH 6.5, being attributable to the π – system of the benzene ring. The positions of the bands depended on the ionization state and on the nature of the subsistent, i.e., their electron donating or withdrawing nature. GA exhibited one band at 215 nm and another at 265 nm, whilst CA exhibited one at 295 nm and another at 315 nm. After 24 h of laccase incubation, both the CA and GA spectra changed. The GA bands at 215 and 265 nm decreased, broadened, and shifted to 257 and 300 nm, respectively. Both shifts are associated with the n–π^* transition due to the C–C and C–O–C coupling, indicating the occurrence of a reaction involving phenolic side-chains (Pati, Losito, Palmisano, & Zambonin, 2006). Similar changes in the CA spectra were observed, i.e., bands at 295 nm and 315 nm decreased, broadened, and the second band shifted to 325 nm, indicating further polymerization with increased conjugation length and a reduction in energy for the electron transitions during light absorption.

In order to spectroscopically identify the laccase-catalyzed phenolic oxidation in the presence of chitosan, CA- and GA-laccase functionalized and non-functionalized chitosan samples of 0.5 (*w*/*v*) % were dissolved in 0.1 M HCl. As shown in Fig. 2, the spectral features of the functionalized chitosan at pH 4.5 were similar to the spectra of laccase polymerized phenolic acids at pH 6.5 (Fig. 1), suggesting the presence of similar chromophores and/or structural analogies. The shifted and decreased bands can be attributed to the lesser amount of energy required for the π–π^* and n–π^* transition or the electron delocalization of increased CA and GA oligomeric/polymeric structures conjugated by the C–C and C–O–C coupling reactions. In addition, the appearance of a band at around 350 nm, consistent with the reaction between *o*-quinones formed by laccase and the amino groups of chitosan (Sousa et al., 2009) was unobserved. Chitosan exhibits pH-dependent solubility. Before complete dissolution, chitosan undergoes pH-sensitive swelling due to the protonation of the amino group under low pH conditions. Upon dissolution at pH 4.5, the free amino groups are fully protonated, resulting in non-reactive amino groups towards *o*-quinones.

In order to assess the GA and CA redox properties, their availability to transfer electrons to the electrode during laccase oxidation, was investigated by cyclic voltammetry. As presented in Fig. 3, the polymerization processes occurred during the laccase monomers' oxidation, which resulted in a decrease in the anodic peak current and the detection of new peaks after 24 h of incubation at pH 6.5.

The fact that CA and GA are structurally similar, both having a catechol skeleton, though GA has an additional hydroxyl group, and CA a vinyl double-bond, led to similar electrochemical oxidation mechanisms via two electron-transfer steps. CA oxidation to CA *o*-quinone gave rise to the high anodic peak at 400 mV, whilst

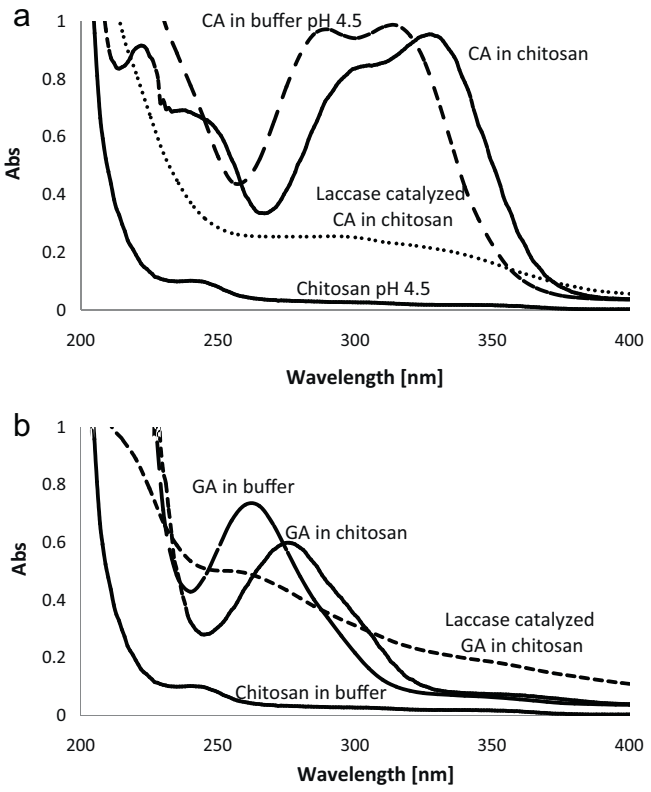

Fig. 2. UV–vis evidence for the laccase catalyzed functionalization of chitosan with (a) CA and (b) GA at pH 4.5.

a reduction of the CA *o*-quinone back to the original CA, produced a cathodic peak at 7 mV on the reverse scan (Fig. 3a). The oxidation involved two sequential single-electron transfer steps, the first one produced phenoxy radicals; and then rapidly followed by the second one leading to the CA *o*-quinone. Because of the aromatization energy contribution and electron donating substituent *ortho* hydroxyl group, the second electron transfer step was significantly easier, but separately non-detectable on the GCE. The presence of laccase in the CA solution led to the appearance of the two new timely decreased anodic peaks seen at 420 mV and 740 mV, and the new cathodic peaks at 210 mV and −20 mV after 24 h of incubation, indicating on the redox reversibly of newly synthesized oligomeric/polymeric compounds, being also confirmed by SEC (Table 1). In the case of GA (Fig. 3b), two anodic peaks appeared because of the higher-reduction potential and low O–H bond dissociation enthalpy of the three-hydroxyl groups on the benzene ring (Pasanphan & Chirachanchai, 2008). In accordance with Gunckel et al. (1998) and Yoshida et al. (1989), the first anodic peak at 400 mV corresponded to the oxidation of the first phenolic hydroxyl group of GA, and the second oxidation peak at 800 mV to the second electron transfer of *ortho* hydroxyl groups. On the other hand, currents beyond 700 mV may be due to the third (*meta*) hydroxyl group or due to the structures of the new oxidation products of chatechols created by non-enzymatic electrode-oxidation (Hotta, Sakamoto, Nagano, Osakai, & Tsujino, 2001). During the reverse sweep, no distinct reduction wave was observed, showing that GA was irreversibly oxidized at the GCE. The GA oligomeric/polymeric products generated after 24 h (Table 1) showed two decreased irreversible electrodic processes, a broad one at a potential from 350 mV to 450 mV, and another at 700 mV.

Cyclic voltammetry measurements showed that, when GA and CA are oxidized by laccase at pH 6.5, they exhibit larger numbers of electrons involved in their oxidation than those expected from their monomer structures (i.e. the number of –OH moieties).

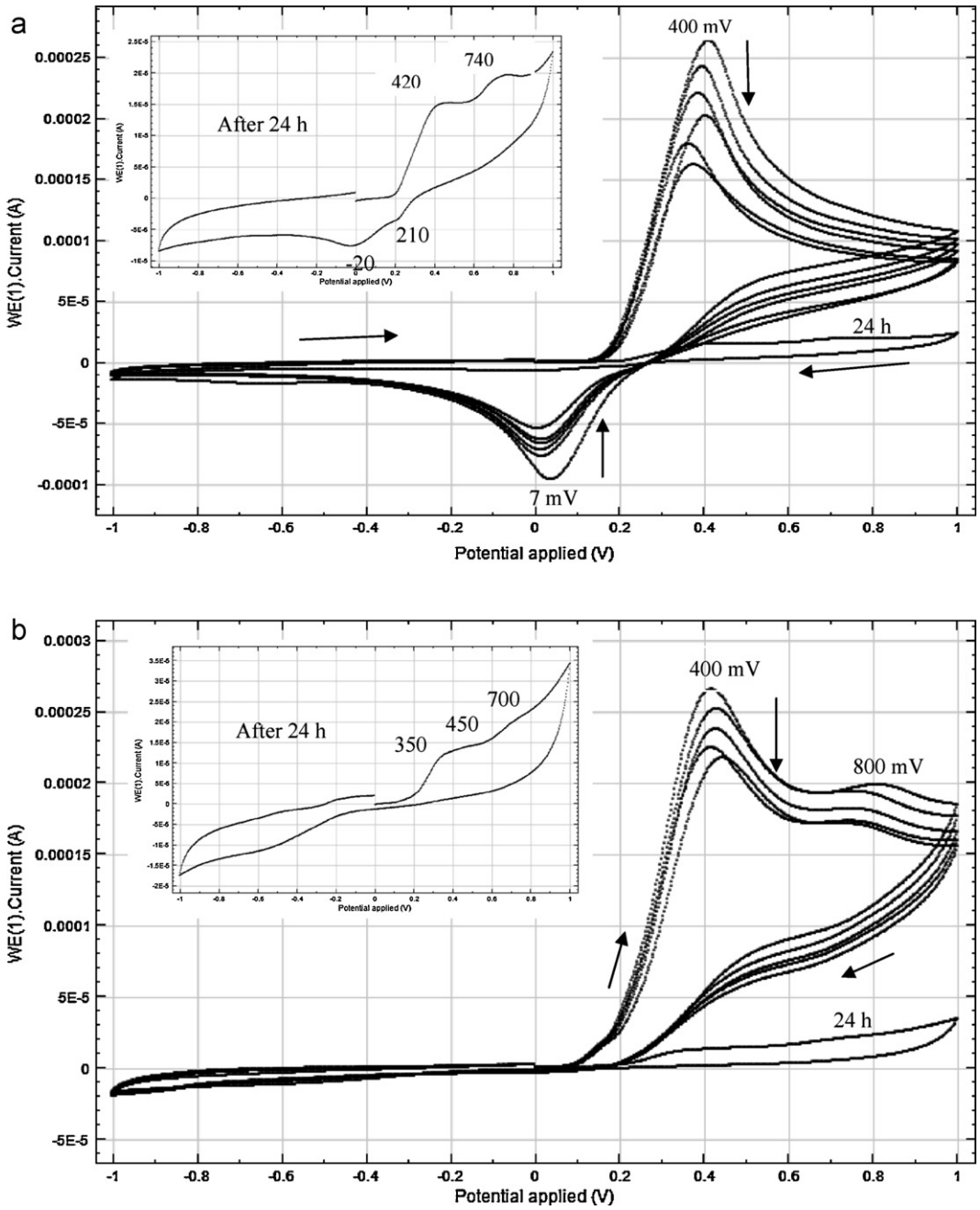

Fig. 3. Time-decreasing cyclic voltammograms of (a) CA and (b) GA in the presence of laccase at pH 6.5.

Through the coupling reaction, –OH moieties are regenerated, so that the synthesized oligomers/polymers possessed an increased number of –OH moieties. From an electrochemical viewpoint, it can be inferred that the redox potential value of phenolic monomers/oligomers should correlate with their antioxidant activity (Hotta et al., 2001). In addition, their reversibility allows the recovery of the –OH moieties when conditions are changed, and enables a lasting capacity for radical scavenging activity.

Table 1
SEC analysis (average molecular weights M_w, retention time R_t and peak areas) of phenolic oligomers/polymers obtained by laccase oxidation at pH 6.5.

	M_w (g/mol)/R_t (min)/area (%)				
M_w (CA) = 180.16 g/mol	665.12	803.34	1394.94	2855.87	3921.53
	5.9595	5.962	5.418	5.28	5.16
	37.56%	7.2%	50.55%	2.84%	1.42%
M_w (GA) = 170.12 g/mol	408.07	684.5	1394.9	2539.4	4098.5
	5.9595	5.709	5.419	5.28	5.15
	6.66%	26.17%	60.36%	3.61%	1.61%

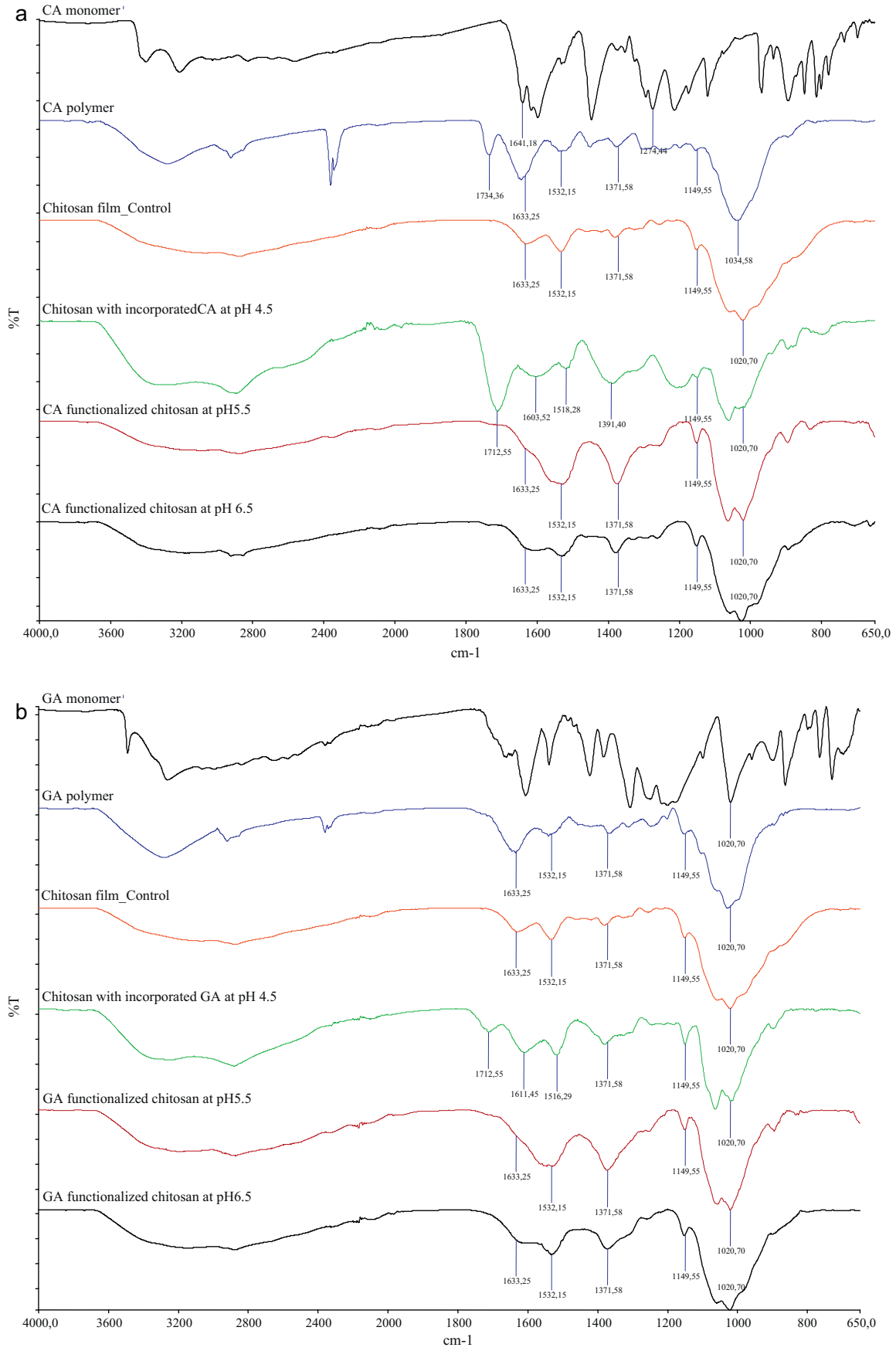

Fig. 4. FTIR spectra of chitosan, CA and GA monomers and theirs oligomers/polymers polymerized by laccase-induced oxidation, and chitosan functionalized with laccase-oxidized (a) CA and (b) GA substituents at different pHs.

3.2. Laccase-catalyzed functionalization of chitosan with GA and CA

FTIR and ^{1}H NMR spectroscopies were used in order to characterize the reaction mechanisms of laccase-oxidized phenolic acids and their produced oligomers/polymers with chitosan polymer, under different pH conditions.

Fig. 4 presents the FTIR spectra of the CA and GA functionalized chitosan films (at pHs 4.5, 5.5 and 6.5), phenolic monomers and their polymerization products synthesized by laccase oxidation at pH 6.5, 30 °C for 24 h. The CA and GA oligomers/polymers spectra exhibited different profiles compared to their monomers. Both of them showed typical polyphenolic characteristics with the existence of a phenolic ring –OH stretching within the 3200–3550 cm^{-1} and –OH in plane bending at 1365 cm^{-1}, an aromatic ring C=C stretching within the 1450–1600 cm^{-1}, and C–O/C–C stretching vibrations within the 1200–1300 cm^{-1}. As a consequence of the high amount of free and H-bonded –OH groups, absorption bands within the 3200–3550 cm^{-1} appeared as one broad summary band. Thus, the bands' resolutions were improved by their deconvolution from the background scattering, using a Gaussian function curve-fitting analysis, performed on Peak Fit® v4.12 software. For confirmation of the results, the second derivative was inspected and local minimums used for the determination of the bands' positions. As presented in Table 2, the GA monomer absorption peak at about 1020 cm^{-1} shifted and split into two absorption bands in the corresponding oligomers/polymers spectra. The first one at about 1025 cm^{-1} can be attributed to the C–O stretching vibration indicating the extended polymerization (Yamada, Abe, & Tanizawa, 2007), and the second one at about 1069 cm^{-1} indicating the phenolic acids' C–C linkages. In the case of the CA oligomers/polymers spectra, only the C–C linkages could be detected, attributed to the absorption peak at about 1041 cm^{-1}. Furthermore, the vibration band of carbonyl stretching, corresponding to the ester bonds, appeared within the 1735–1750 cm^{-1} in both the oligomers/polymers spectra, whilst it was missing in the monomers' spectra. This can be attributed to non-enzymatic esterification employing hydroxyl and carboxyl groups of the phenolic acids. In addition, the disappearances of the characteristic fingerprint regions for both monomers can be observed in the polymers spectra.

The laccase can oxidize phenols and convert them into reactive *o*-quinones, which undergo oligomer-forming reactions with other quinones, as well as a complex set of non-enzymatic reactions with nucleophilic amine groups of chitosan (Muzzarelli, Littarru, Muzzarelli, & Tosi, 2003). The chemistry of the reaction is not well understood, but it is expected that quinones are covalently conjugated to free amine groups through the Schiff-base and/or Michael-type reactions mechanisms (Sampaio, Taddei, Monti, Buchert, & Freddi, 2005).

The spectra of the untreated chitosan (Fig. 4) showed a broad absorption band within the 3200–3500 cm^{-1}, attributed to –OH and –NH stretching and bands at about 1632 cm^{-1}, 1555 cm^{-1}, and 1380 cm^{-1} attributed to the amide I, II, and III modes of the residual N-acetyl groups, respectively. The absorption band at 1150 cm^{-1} corresponding to the anti-symmetrical stretching of the C–O–C bridge and at 1080 cm^{-1} corresponding to skeletal vibration involving C–O stretching, are characteristics of a chitosan saccharide structure (Muzzarelli, Tanfani, Scarpini, & Laterza, 1980; Sousa et al., 2009). The spectra of the CA and GA laccase functionalized chitosan films obtained at pHs 6.5 and 5.5 showed the most significant changes. These involved shoulder of the 1650 cm^{-1} band, attributed to the C=N stretching mode of imines (Sampaio et al., 2005), supporting the occurrence of a Schiff-base reaction. Analogously, the Michael addition-type reaction can be supported by the trend of the conversion of some primary amines into secondary amines, NH stretching at 3300–3500 cm^{-1} and NH bending around 1530 cm^{-1}, which became broader due to an increase in the aromatic and non-aromatic stretching hydroxyl groups of phenolic acids (Mohammed-Ziegler & Billes, 2002), providing direct information on its structural changes occurring during the reaction. At pH 4.5, chitosans amine groups, being fully protonated, are not nucleophilic and therefore cannot react with electrophiles,

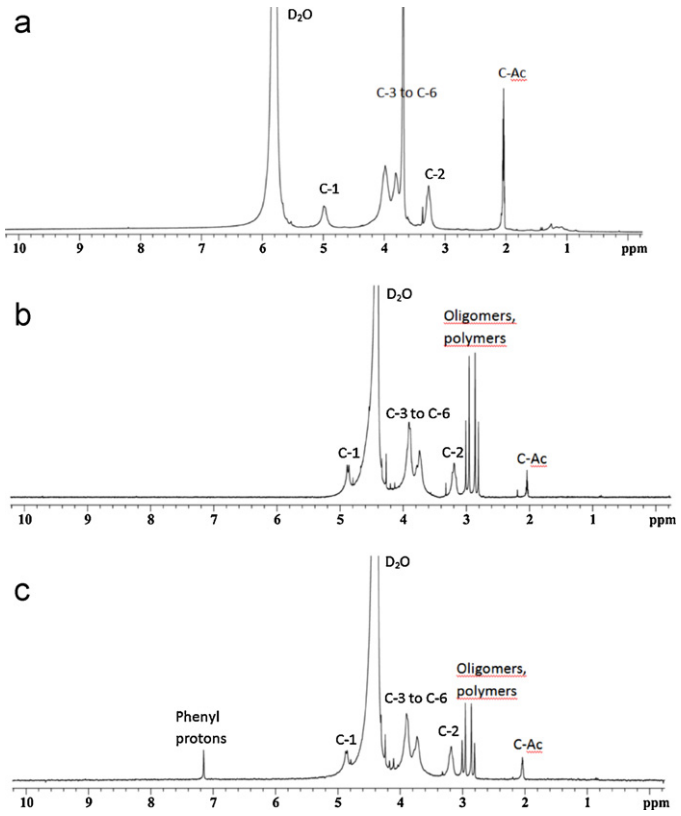

Fig. 5. ^{1}H NMR spectra of (a) chitosan, (b) CA-functionalized chitosan at pH 4.5 and (c) GA-functionalized chitosan at pH 4.5.

Table 2
Characteristic FTIR vibrations of phenolic monomers and their oligomeric/polymeric products obtained after incubation with laccase at pH 6.5.

FTIR vibration	GA monomer		GA polymers		FTIR vibration	CA monomer		CA polymers	
	Peak (cm^{-1})	Abs	Peak (cm^{-1})	Abs		Peak (cm^{-1})	Abs	Peak (cm^{-1})	Abs
νOH(O9–H16)	3487.7	0.16	3481.6	0.04	νOH(O7–H18)	3429.4	0.11	3510.0	0.01
νOH(O10–H17)	3413.4	0.10	3409.0	0.07	νOH(O8–H17)	3399.4	0.13	3399.8	0.02
νOH(O11–H18)	3522.7	0.01	3535.0	0.04	νOH(O13–H21)	3208.8	0.18	3290.0	0.03
νOH(O14–H15)	3256.0	0.33	3267.6	0.11					
νCO (C7=O13)			1737.7	0.01	νCO (C11=O12)			1739.8	0.03
νCC (in ring)	1660.0	0.02	1654.5	0.10	νCC(C9=C10)	1641.0	0.35	1649.5	0.05
(C–O; C–C)	1018.9	0.49	1025.6	0.24	(C–O; C–C)			1040.6	0.09
			1069.2	0.18					

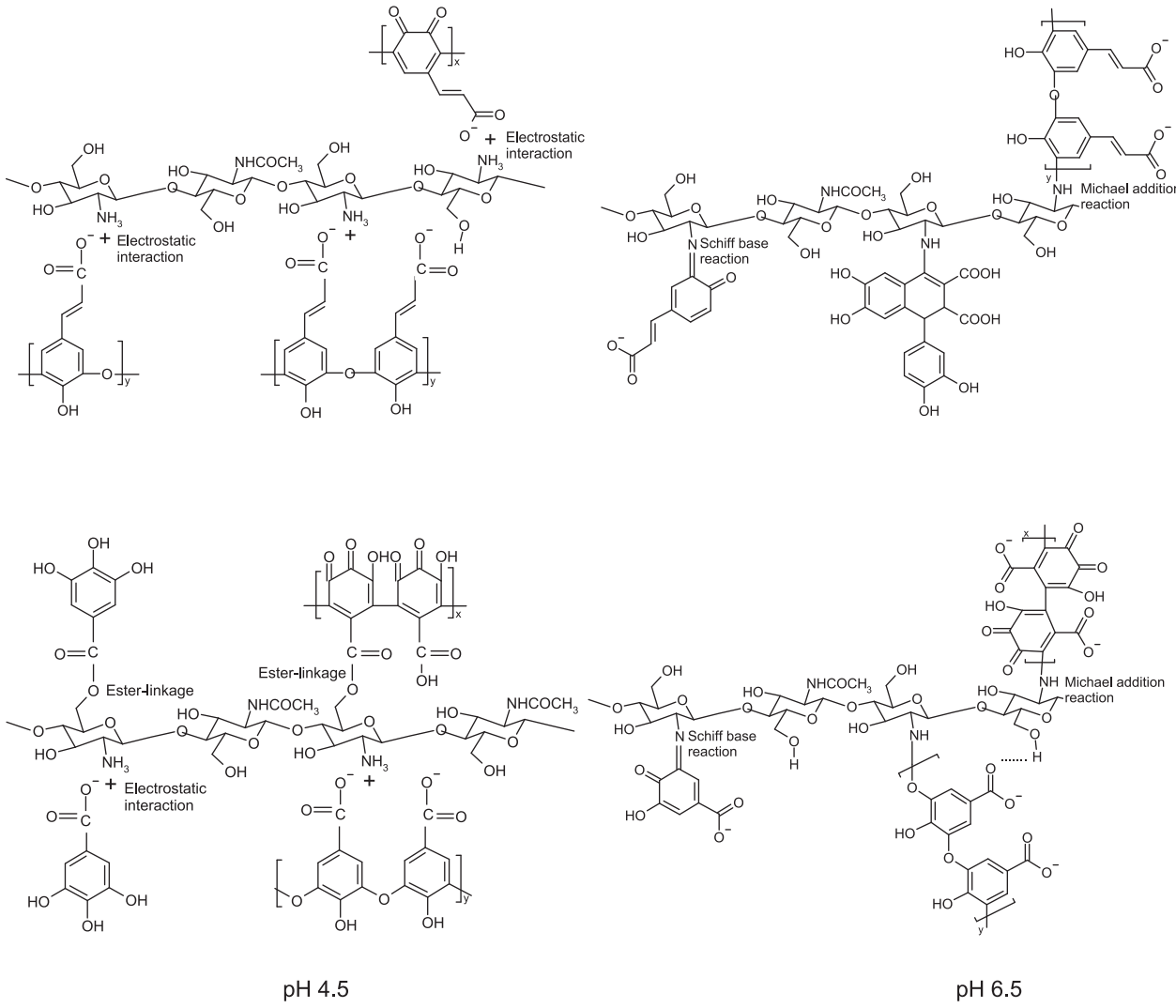

Fig. 6. The proposed functionalization mechanisms of chitosan with CA (above) and GA (below) obtained by laccase catalyzed reactions at different pHs.

including laccase-generated quinones (Osman & Arof, 2003). However, both, the GA- and CA-chitosans functionalized at pH 4.5, revealed new bands located at around 1715 cm^{-1}. Whilst GA band is assigned to the ester linkages between chitosan and non-dissociated GA (pK_a of COOH in GA is 4.41), CA band corresponds to the ester linkages between CA polymerized products. The partially dissociated GA and fully dissociated CA (pK_a of COOH in CA is 4.04) and their oligomers/polymers also formed electrostatic interactions with the positively charged amino group of the chitosan, and additional inter- and intramolecular hydrogen-bonds between the bulky groups of the benzene ring and chitosan network.

^{1}H NMR was used as another technique for demonstrating the functionalization mechanisms of chitosan with GA and CA. The ^{1}H NMR spectra of non-functionalized chitosan polymer (the control), presented in Fig. 5a, shows characteristic peaks of glucosoamine residues at 5.0 ppm for the anomeric proton on C-1 and at 3.1 ppm for the proton on C-2. The peak for the methyl protons of the *N*-acetylglucosamine residues (C-Ac) was at 2.0 ppm, and peaks between 3.4 and 4.1 ppm for protons were on C-3, C-4, C-5, and C-6. The GA-functionalized chitosan polymers at pH 4.5 showed a new peak at 7.1 ppm (Fig. 5c) belonging to the phenyl protons, which confirmed the non-enzymatic conjugation of GA monomer onto the chitosan polymer via its hydroxyl groups on C-6 (Pasanphan & Chirachanchai, 2008). The numerous peaks between 2.8 and 3.4 ppm suggest that the solution is a mixture of multiple products, e.g., includes various oligomers/polymers of GA. The same peaks between 2.8 and 3.4 ppm were present for CA-functionalized chitosan at pH 4.5, however a peak belonging to the phenyl protons between 6 and 8 ppm was unobserved (Fig. 5b). The ^{1}H NMR spectra also confirmed that GA- and CA-functionalized chitosans may contain more than single types of GA and CA oligomers/polymers, which is in agreement with the SEC results. However, the weight of evidence supports the conclusion that, at pH 4.5, the GA fractions are covalently grafted with the chitosan polymer by ester bonds, whilst only electrostatic interactions between chitosan and CA fractions can occur.

The proposed functionalization mechanisms of chitosan with CA and GA, obtained by laccase-catalyzed reactions at different pHs are presented in Fig. 6.

3.3. Antioxidant activity of GA- and CA-functionalized chitosan

All the tested samples showed relatively strong inhibitory activity towards the ABTS cation radicals. However, the highest was for the GA monomer and its oligomers/polymers, which correlates with redox-ability determination. It is well-known that the antioxidant activities of chatechols and their substrates depend on both the total number and the configuration of H-donating hydroxyl groups at specific positions on the benzene ring.

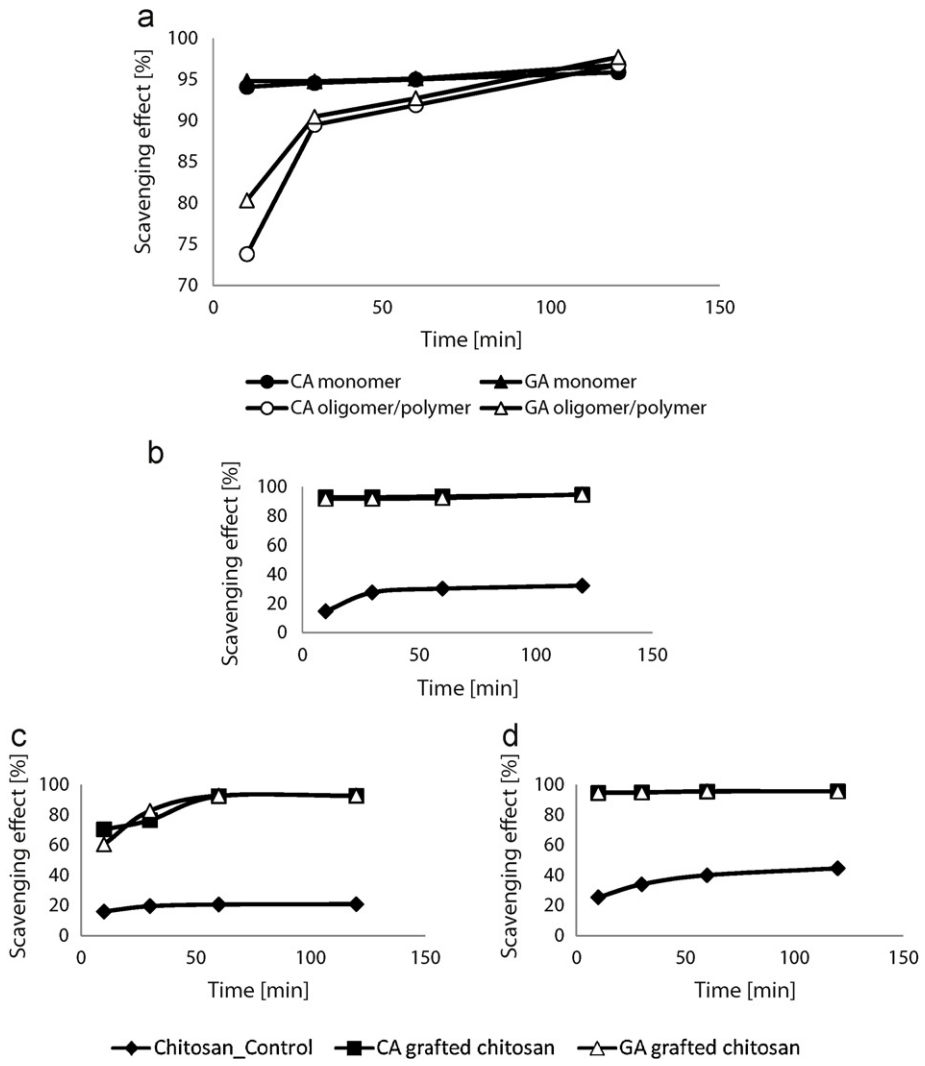

Fig. 7. Time-dependent antioxidant activity of (a) phenolic monomers and their laccase-induced reaction products synthesized at pH 6.5, and chitosan functionalized with laccase-oxidized GA and CA at pH 4.5 (b), and on chitosan film surfaces at pH 5.5 (c) and 6.5 (d).

However, an increase in antioxidant activity could be observed for both, the monomeric and oligomeric/polymeric compounds over time (Fig. 7a). This was possibly because of the reproduced oxidizable –OH moieties in their polymerized products, higher radical stability arising from greater electron delocalization, and the branched structure of the oligomeric/polymeric products. Furthermore, due to the steric hindrances of the branched chain, the mobility of the free-radicals was lower, and additional time was required to enter the active site.

CA and GA functionalized chitosan films exhibited much higher ABTS radical cation scavenging activities compared to the native chitosan film (Fig. 7b–d). The highest activity was found to be for chitosan modified at pH 4.5, where part of GA compounds were grafted through a carboxylic acid tail, whilst the other part of the dissociated carboxylic group formed electrostatic bonds with the positively charged amino group of the chitosan. This allowed for a freely reachable benzene ring with –OH moieties for the radicals' stabilization. On the other hand, when functionalization was performed at pHs 5.5 and 6.5, CA and GA were covalently linked by –NH– linkage on the chitosan macromolecule, and the availability CAs and GAs –OH moieties was reduced due to steric obstruction. Furthermore, at pH 5.5, only around 9% of chitosan amino groups were available for grafting, which is associated with slower scavenging activity but comparable with others after 1 h.

3.4. Antimicrobial activity of GA- and CA-functionalized chitosan

Table 3 presents the antimicrobial/antifungal activities for GA- and CA-functionalized chitosan films against *E. coli*, *S. enterica*, *L. monocytogenes* and *C. albicans*. A reference chitosan film exhibited relatively high antimicrobial activity compared to all the modified

Table 3
Antimicrobial activity of chitosan (reference) and chitosan functionalized with laccase-oxidized GA and CA at different pHs and procedures (within the chitosan solution – at pH 4.5, on the chitosan surface – at pH 5.5 and pH 6.5).

Sample	Reduction of bacteria and fungi (%)			
	E. coli G–	*S. enteric* G–	*L. monocytogenes* G+	*C. albicans*
pH 4.5				
Reference	54	0	56	39
CA	57	0	68	60
GA	97	0	44	56
pH 5.5				
Reference	89	43	89	61
CA	34	49	20	57
GA	53	50	13	70
pH 6.5				
Reference	99	31	98	88
CA	3	31	14	80
GA	13	28	10	87

ones. This is usually described as due to the greater availability of positively charged amino groups in the former, which makes chitosan a bioadhesive for negatively charged surfaces such as bacterial cell, being however dependent on both the modification of chitosan and the microorganism used. By chitosan functionalization at pHs 5.5 and 6.5, the availability of free amino groups was reduced, which resulted in a reduction in antimicrobial activity against *E. coli* and *L. monocytogenes*. On the other hand, when comparing the reference chitosan prepared in a solution at pH 4.5 with the functionalized chitosan at pH 4.5, the functionalized chitosan exhibited increased activity against *E. coli* and *L. monocytogenes*. Thus, GA incorporation onto the chitosan polymer completely inhibited the *E. coli* microbial growth. According to the postulated hypothesis, the phenolic hydroxyl group (Ultee, Bennik, & Moezelaar, 2002) acts as a proton exchanger, and can reduce the pH gradient across the cytoplasmic membrane. The resulting collapse of the proton-motive force, and depletion of the ATP pool, eventually leads to the death of *E. coli*. In the case of antifungal activity against *C. albicans*, the functionalized chitosan films prepared at pHs 5.5 and 6.5 were more effective than those films prepared at pH 4.5. This might be due to the fundamental differences in the microbial cell-walls between fungi and bacteria. The fungal cell-walls are composed of chitin and other polysaccharides such as β-1,3-glucan (zymosan) and mannose-containing glycoproteins, whilst the gram-positive bacteria cell-walls are composed of peptidoglycan, being an acidic polymer such as teichoic acid, lipo teichoic acid, and teichuronic acid. Therefore, it is possible that the functionalized chitosan films can be easily absorbed onto the fungal cells' surfaces, leading to leakages of proteinaceous and other intracellular constituents. *S. enterica* was the most resistant of the tested microorganisms or, with insufficient reduction, in all the tested modified chitosan films. These findings match with previous works in which gram-negative bacteria seemed to present higher resistance against chitosan, probably due to the outer membrane that they show (No, Kim, Lee, Park, & Prinyawiwatkul, 2006).

4. Conclusion

The presented paper demonstrates the laccase-mediated and pH-dependent functionalization of antimicrobially active chitosan with CA and GA as a new strategy to realize the modulation of synergistic antioxidant activity and antimicrobial activity of chitosan-based materials.

The laccase-initiated oxidation of phenolic acids at different pHs in the presence and absence of chitosan, was followed and analyzed using UV–vis spectroscopy and cyclic voltammetry, whilst the synthesized oligomers/polymers were determined by HPLC-SEC and identified using FTIR and ^{1}H NMR spectroscopy's. The ability of functionalized chitosan materials to inhibit $ABTS^{\bullet+}$ radicals was determined, and tested against *E. coli*, *S. enterica*, *L. monocytogenes* and *C. albicans* to characterize their antimicrobial properties.

The analysis indicated that the laccase catalyzed oxidation of GA and CA resulted in C–C and C–O–C coupling reactions, and formation of new phenolic oligomeric/polymeric structures with stronger free-radical scavenging activities when compared to theirs monomers. In addition, *o*-quinone-amino coupling reactions via Schiff-base and Michael addition mechanisms, ester-formation, and electrostatic interactions of phenolic oligomers/polymers with chitosan were identified, depending on the type of phenolic acid used and the pH medium, indicating that the chitosan antimicrobial and antioxidant activities can be varied depending on the type and quantity of its functionalization with phenolic acids and the degree of their polymerization. It is clearly shown that the laccase-mediated strategy of chitosan functionalization with phenols may provide a novel way of preparing and exploiting many other multifunctional chitosan-based materials for wider applications, e.g., in pharmaceutical, cosmetic and food industry.

Acknowledgements

This work was supported by the Ministry of Higher Education, Science and Technology [Eureka, grant no. E!14952]. We are also grateful to Miha Friedrich and Prof. Dr. Janez Plavec from the National Chemistry Institute in Ljubljana for the performance of ^{1}H NMR analysis.

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