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Enzymatic gelation of the natural polymer chitosan

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Abstract

The biopolymer chitosan was modified using the enzyme tyrosinase to convert a low molecular weight phenolic substrate (*p*-cresol) into a reactive *o*-quinone that undergoes subsequent reaction with chitosan. Spectroscopic data support the conclusion that the phenolic groups are covalently grafted at the amine site of chitosan although specific linkages could not be established due to the complexity of the chemistry. When semi-dilute chitosan solutions (<1%) were incubated with *p*-cresol and tyrosinase, the steady shear viscosity was observed to increase dramatically while oscillatory shear measurements indicated that the chitosan solutions had been converted into a gel. Specifically, dynamic oscillatory shear measurements showed that enzymatic reaction resulted in large increases in the complex viscosity (η^*), and storage and loss moduli (G' and G''). Further, η^* for the cresol-modified chitosan was observed to decrease with increasing frequency (ω), while G' and G'' became independent of ω and the loss tangent (tan δ) became less than 0.1. Incubation of these gels with the hydrolytic enzyme chitosanase showed that the cresol-modified chitosan remained biodegradable.

Keywords: Natural polymer chitosan; Tyrosinase; Chitosanase

1. Introduction

There is a growing interest in exploiting renewable resources for the production of biodegradable polymers [1]. Chitosan, which is obtained by N-deacetylation of chitin, has attracted some attention since chitin is the second most abundant polysaccharide. Typically, chitosan is produced from wastes generated from the crustacean processing (shrimp and crabs), but it is also possible to obtain from the chitin component of fungal cell walls. In addition to being derived from renewable resources, chitosan offers unique physical, chemical and biological properties, which have been studied for various applications (see Refs. [2,3]).

The high content of primary amino groups makes chitosan unique and confers two important characteristics to this biopolymer. First, Scheme 1 shows that the amino groups are basic and at moderately low pH (<6.3), chitosan is protonated and the resulting cationic polyelectrolyte is water soluble. Water solubility is unusual for β-1,4 linked polysaccharides. For instance, chemical modification (e.g. with carboxymethyl or hydroxyethyl groups) is required to confer water solubility to the β-1,4-linked polysaccharide cellulose.

The second important characteristic of chitosan's amino groups is their nucleophilicity. At high pH (>7), Scheme 1 shows the amino groups are deprotonated and the unshared electron pair can undergo a variety of reactions. The reactivity of these amino groups can be exploited to chemically modify chitosan under mild conditions. For instance, several groups have generated modified chitosans by exploiting reactions between chitosan's amino groups and aldehydes [4-7] or acid anhydrides [8-11]. Because of the relatively mild conditions used for these reactions, modification is typically confined to chitosan's amino group [6,8,11,12]. This site-selectivity suggests the potential for better controlling the functional properties of modified chitosans. In contrast, cellulose modification typically requires strongly basic conditions and reactions can occur at hydroxyl groups in the 2, 3 and 6 positions (see Ref. [13]).

Previously, we examined an enzymatic method for modifying chitosan using the enzyme tyrosinase that catalyzes the oxidation of phenolic substrates into *o*-quinones [14–16] (Scheme 2). These quinones are electrophilic and are known to undergo a variety of non-enzymatic reactions including reactions with amino groups. In nature, reactions involving enzymatically-generated quinones occur during melanization, enzymatic food

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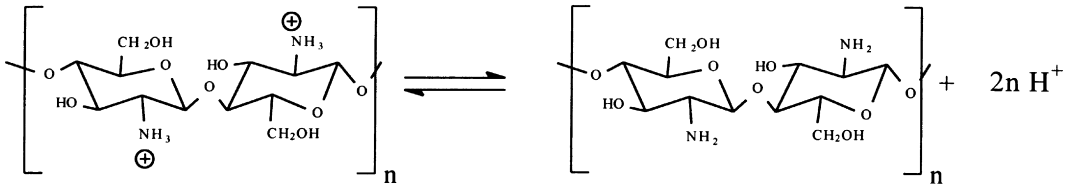


- Polycationic
- Water-soluble
- Unreactive

- Neutral
- Insoluble in water
- Nucleophilic and reactive

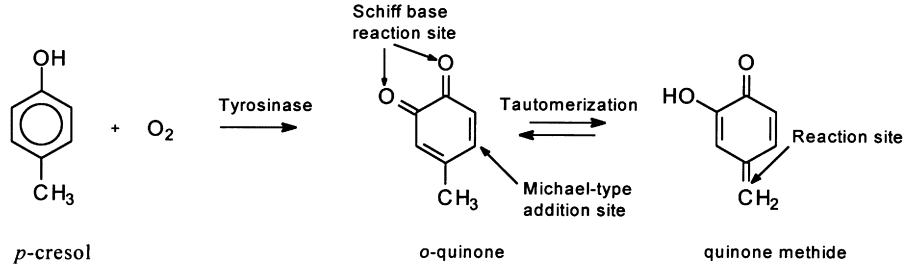
Scheme 1.

browning and insect sclerotization (i.e. hardening of the "shell") [17-21]. A potential advantage of tyrosinase for chitosan-modification is that the enzyme functions to activate a phenolic substrate into a reactive *o*-quinone, and this in situ activation may offer safety advantages over modification methods that require the use of reactive reagents. Another potential advantage is that the tyrosinase-catalyzed chitosan modification may have generic utility because these enzymes are robust, only require molecular oxygen as a co-substrate, catalyze reactions under mild conditions, and have a broad substrate range for phenols. In addition to reacting with a range of monomeric phenols, tyrosinase is known to react with oligomeric and polymeric substrates. For instance, various groups have studied the reaction of tyrosinase with tyrosine or dihydroxyphenylalanine (DOPA) residues of peptides and proteins [22–27]. Also, Kaleem et al. [28] and Muzzarelli and coworkers [29,30] chemically grafted phenolic moieties onto natural polymers (gelatin or chitosan) and reported that tyrosinase was capable of reacting with these grafted phenolic moieties. Finally, Shao et al. [31] reported that tyrosinase can react with phenolic moieties on the synthetic polymer, polyhydroxystyrene (polyvinylphenol).

One obvious limitation to the use of tyrosinase is that the freely diffusible reactive intermediate (i.e. *o*-quinone) can undergo a variety of reactions and controlling or even characterizing the subsequent quinone reactions can pose a substantial challenge [18]. For instance, quinones and amines can react to form both Schiff bases and Michael-type adducts, and quinones can also undergo polyphenol-forming reactions. As observed in previous studies [16] and reported here, it seems likely that enzymatically-generated quinones preferentially react with the 2-amino group of chitosan. However, the grafted quinoid moieties may not be a single species, but rather an array of monomeric and oligomeric phenols that could be grafted through differing chemical linkages.

Despite the complexity of the chemistry, tyrosinase-catalyzed reactions may be practically exploited to confer important functional properties. For example, there has been considerable study of how tyrosinase-catalyzed reactions of tyrosine- or DOPA-rich proteins can yield moisture-resistant adhesives [24,26,27,32]. Recently, we showed that tyrosinase-catalyzed modification of chitosan with the phenolic natural product, chlorogenic acid, resulted in a modified chitosan that was soluble under acidic and basic conditions and had a window of insolubility at neutral pH [16].

The goal of the work reported here was to demonstrate that chitosan gels can be formed enzymatically by incubating semi-dilute chitosan solutions (<1% chitosan) with tyrosinase and the simple phenolic substrate *p*-cresol. Scheme 2 suggests that the gel formation could result from chemical cross-linking because the tyrosinase-generated *o*-quinones have multiple sites for reaction with chitosan's amino groups. Roberts and Taylor [7] studied a chemical method for converting chitosan solutions into gels. In their work, they used glutaraldehyde, a difunctional agent capable of chemically cross-linking chitosan. It is also possible that enzymatically-modified chitosan could form physical gels. As suggested in Scheme 1, physical gelation of chitosan can be achieved by simply raising the pH of a chitosan solution such that the amino groups become deprotonated and electrostatic repulsions of the polymer chains



Scheme 2.

are diminished [33]. Physical gelation has also been reported when chitosan is chemically modified with mono-functional aldehydes [4] and acid anhydrides [9,10,34,35] that are incapable of chemical cross-linking. Cairns et al. [33] reported that gels formed by N-acetylation of chitosan had X-ray diffraction patterns suggesting that junction zones in these gels may result from the formation of crystalline regions. Physical gels could also be formed if hydrophobic interactions are responsible for the formation of network junctions. Kjoniksen et al. [36,37] reported such gels with hydrophobically-modified chitosans. Finally, it is possible that a combination of chemical and physical interactions could result in gel formation [38].

2. Materials and methods

Chitosan, *p*-cresol, mushroom tyrosinase and chitosanase were obtained from Sigma Chemical Co. Mushroom tyrosinase and chitosanase were reported by the manufacturer to have specific activities of 3,000 and 208 U mg^{-1}, respectively. Spectra/Por pure regenerated cellulose film was purchased from Fisher Scientific. Deuterium oxide (D_2O) was obtained from Cambridge Isotope Laboratories. All other chemicals were obtained from Fisher Scientific.

Chitosan solutions (1.6 w/v%) were prepared by adding 1.6 g chitosan to 100 ml deionized water and slowly adding hydrochloric acid (2 M) to attain a final pH of 2–3. After mixing overnight, undissolved material was removed from the viscous chitosan solutions by vacuum filtration.

Chitosan films were prepared by diluting the 1.6% chitosan solution to 0.5–0.6% and pipetting 2 ml solution into 3.5 cm petri dishes. The solution was allowed to dry overnight in the oven at 60°C. The films, thus formed, were removed from the petri dishes and immersed in 1 M NaOH for 4–5 h. The films were then thoroughly washed with deionized water and stored in phosphate buffer (100 mM, pH 6.5). Cellulose films were also washed several times with deionized water and stored in phosphate buffer.

Heterogeneous reactions with chitosan and cellulose films were conducted by incubating the films for 1 or 2 h in a 20 ml phosphate buffer (100 mM; pH 6.5) containing *p*-cresol (1.8 mM) and tyrosinase (20 U ml^{-1}). After reaction, the films were washed extensively with deionized water and then dried. The UV–Vis and IR spectra of the dried films were measured using a Spectronic Genyses 2 spectrophotometer and a Perkin–Elmer System 2000 FT-IR, respectively.

Homogeneous modification reactions were conducted by diluting the 1.6% chitosan solution to 0.16, 0.32 and 0.48% to attain equivalent concentrations of amino groups of 10, 20 and 30 mM, respectively. It should be noted that this equivalent amino group concentration is based on the assumption that chitosan has been completely deacetylated although NMR analysis indicates the degree of deacetylation was between 90 and 95%. Varying levels of *p*-cresol were added to these chitosan solutions and the pH was increased from 5.8 to 6 which is sufficiently low to maintain chitosan in solution. Tyrosinase was then added with the activities varying from 20 to 150 U ml^{-1} depending on the *p*-cresol concentration. These solutions were then incubated overnight at room temperature.

When homogeneously-modified chitosan was characterized by proton NMR, the sample was prepared in the following manner. After reaction, the pH of the reaction mixture was increased to 13–14 using NaOH, and the solution was mixed overnight to dissolve the modified chitosan. To remove low molecular weight solutes (e.g. unreacted *p*-cresol) from the modified chitosan, the pH was re-adjusted to seven using HCl and the polymer-containing precipitate was collected. This precipitate was centrifuged and the insoluble fraction was washed with deionized water. This procedure of dissolving chitosan at high pH, precipitating the polymer at neutral pH, centrifuging and washing was repeated three additional times. The removal of unconverted *p*-cresol from the polymer fraction was monitored by following sharp ^1H NMR signals at 6.7 and 7.0 ppm.

Samples were prepared for ^1H NMR analysis by exchanging the hydrogens on the heteroatoms with deuterium by adding D_2O to the sample and evaporating the solvent several times. ^1H NMR spectra were collected for polymer solutions using a 300 or 400 MHz NMR (General Electric). In cases where an NMR signal was masked by the HOD peak, spectra were collected at 80°C to shift the HOD peak upfield (i.e. from 4.8 to 4.2 ppm).

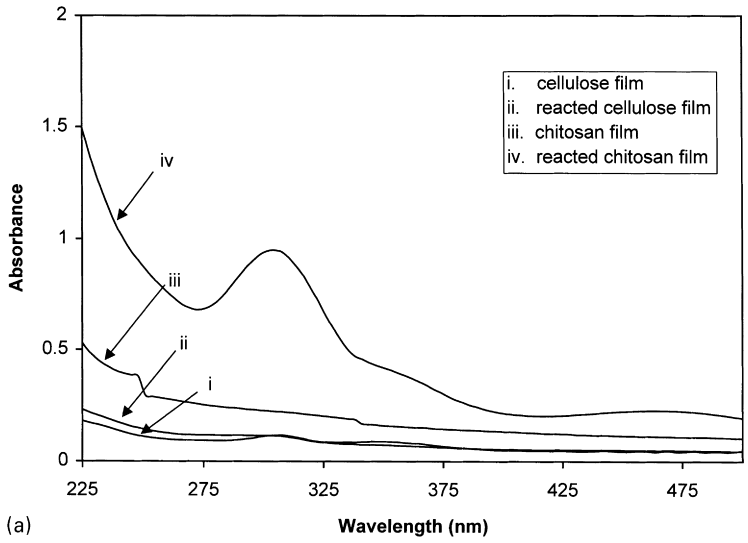
Steady shear viscosities of chitosan solutions and the modified gels were measured using a Brookfield DV II + viscometer with an S25 spindle at 1 rpm.

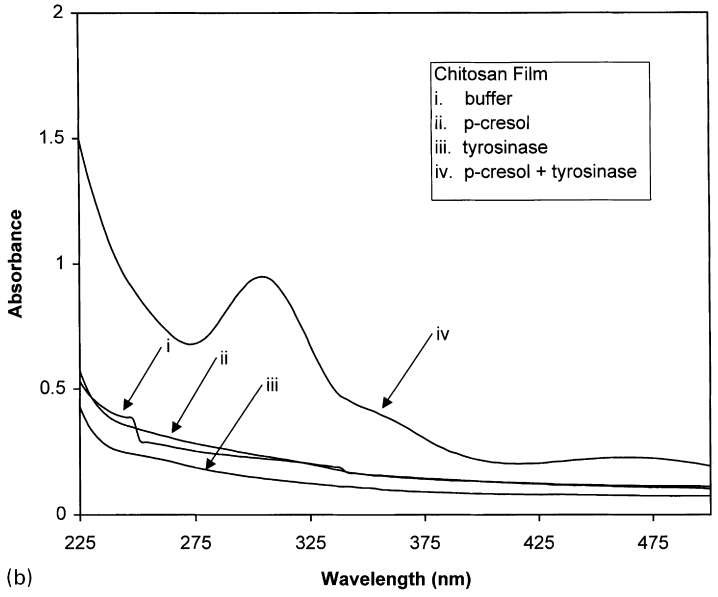
Dynamic measurements on the gels and chitosan solutions were performed using a Rheometrics DSR-200 stress rheometer with a 25 mm cone-and-plate geometry. A strain sweep test at a frequency of 1 rad s^{-1} was performed to determine the linear viscoelastic region, from which an appropriate strain was selected. Using this strain, the storage modulus (G') and the viscous loss modulus (G'') were measured from a constant-strain frequency sweep over a frequency range of 0.1–10 rad s^{-1}. If the time required to measure the dynamic properties at 0.1 rad s^{-1} exceeded 300 s, then a minimum frequency of 0.3 rad s^{-1} was used. This served to restrict the total measurement time to prevent solvent evaporation from affecting the measured properties. The tangent of the phase angle ($\tan\delta$) and the magnitude of the complex viscosity (η^*) were determined from G' and G'' as follows:

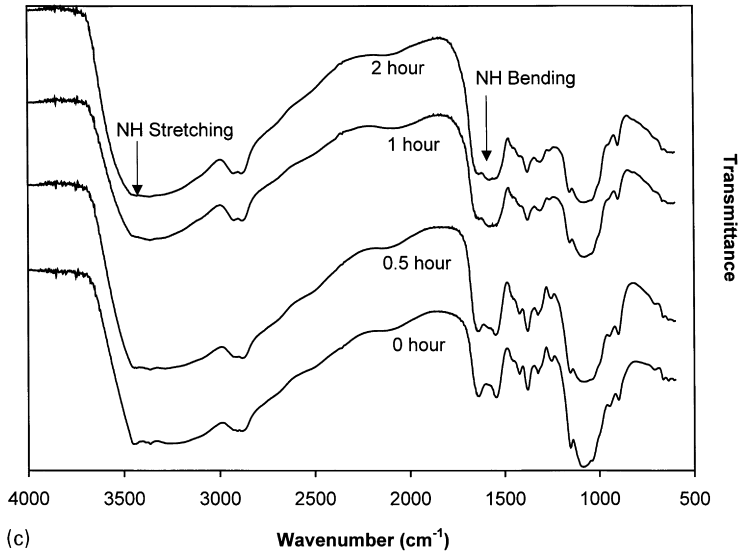
$$\tan\delta = \frac{G''}{G'} \tag{1}$$

$$\eta^* = \sqrt{(\eta')^2 + (\eta'')^2} = \sqrt{(G'/\omega)^2 + (G''/\omega)^2} \tag{2}$$

where ω is the angular frequency.


i. cellulose film
ii. reacted cellulose film
iii. chitosan film
iv. reacted chitosan film
Absorbance
Wavelength (nm)
(a)


Chitosan Film
i. buffer
ii. p-cresol
iii. tyrosinase
iv. p-cresol + tyrosinase
Absorbance
Wavelength (nm)
(b)


2 hour
NH Bending
NH Stretching
1 hour
0.5 hour
0 hour
Transmittance
Wavenumber (cm-1)
(c)

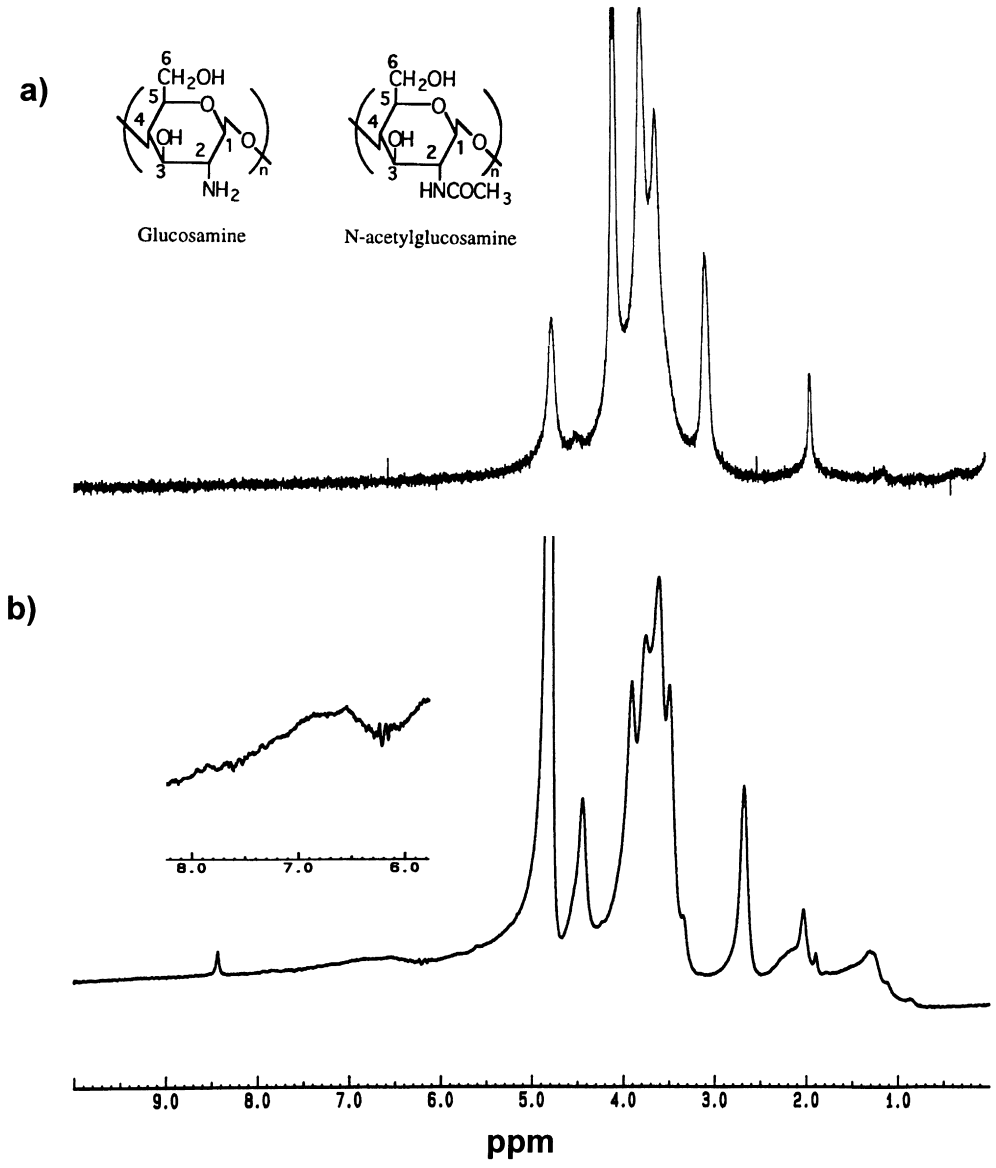


Fig. 2. The solution phase ^{1}H NMR of chitosan and homogeneously modified chitosan. (a) Chitosan (pH = 2) spectrum was collected at 80°C to shift the HOD peak from 4.8 to 4.2 ppm and avoid interference with the peak from the anomeric proton (C-1). (b) Modified chitosan was prepared as described in the text and the spectrum was collected at room temperature and a pH of 12. Insert shows an enlargement of the spectrum between 6 and 8 ppm.

To examine whether the modified chitosan gels could be biologically degraded, 1 U of chitosanase was introduced to a 15 ml of chitosan gel that had been formed by the reaction between a 0.32% chitosan solution and 12 mM *p*-cresol. The gel was stirred with a mechanical stirrer and the steady shear viscosity was measured over time.

3. Results and discussion

3.1. Evidence for enzyme-catalyzed covalent modification of chitosan

Due to the complexity of chemistry, it seems likely that the enzymatically-generated quinones are grafted onto

Fig. 1. The heterogeneous modification of chitosan films. (a) UV–Vis spectra of chitosan and cellulose films which had been incubated for 1 h in a 20 ml phosphate buffer (control) or a buffered solution containing *p*-cresol (1.8 mM) and tyrosinase (20 U ml^{-1}). (b) UV–Vis spectra of chitosan films which had been incubated for 1 h in phosphate buffer, a buffered solution containing either *p*-cresol (1.8 mM) or tyrosinase (20 U ml^{-1}), and buffered solution containing both *p*-cresol (1.8 mM) and tyrosinase (20 U ml^{-1}). (c) FT-IR spectra of chitosan films which had been incubated in phosphate buffer (designated: 0 h) and a buffered solution containing *p*-cresol (1.8 mM) and tyrosinase (20 U ml^{-1}) for varying lengths of time (designated: 0.5, 1 and 2 h). After reaction, the films were thoroughly washed with deionized water and dried prior to measuring the UV–Vis and IR spectra.

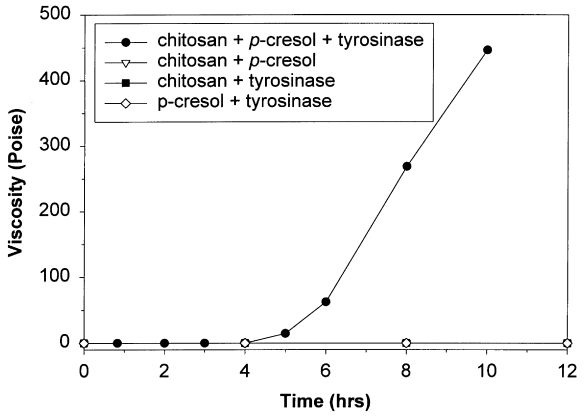


Fig. 3. An enzymatic enhancement of steady shear viscosity. Viscosity was measured for chitosan solutions (0.32%) which were incubated with *p*-cresol (12 mM; symbol not visible), or tyrosinase (80 U ml^{-1}), or both *p*-cresol and tyrosinase. The viscosity of a control solution containing *p*-cresol and tyrosinase in the absence of chitosan was also measured.

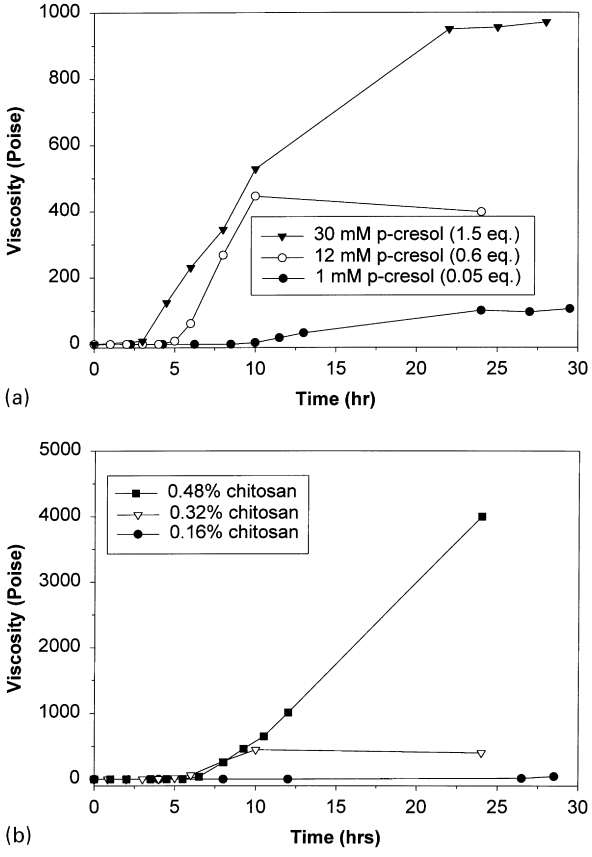


Fig. 4. The effect of varying concentrations on the enzymatic enhancement of steady shear viscosity. (a) Viscosity of chitosan solutions (0.32%) incubated with 1, 12 and 30 mM *p*-cresol (0.05, 0.6 and 1.5 equivalents relative to chitosan amino groups) and 20, 80 and 150 U ml^{-1} tyrosinase, respectively. (b) Viscosity of chitosan solutions incubated with 0.6 equivalents of *p*-cresol (solutions contained: 0.16, 0.32 or 0.48% chitosan; 6, 12 or 18 mM *p*-cresol; and 60, 80, or 100 U ml^{-1} tyrosinase, respectively).

chitosan through various linkages and that the grafted species may include a mixture of monomeric and oligomeric phenolics. Rather than attempting to identify specific linkages and individual moieties, the goal of our chemical analysis was to provide a body of evidence supporting the conclusion that products generated from the tyrosinase-catalyzed oxidation of *p*-cresol are covalently grafted onto the amino group of chitosan. For this goal, we employed various different experimental approaches. Fig. 1(a) shows a comparison between the behavior of chitosan and cellulose films [16,39] before and after incubation with the reaction mixture. Before incubation, both films were clear. After incubation with tyrosinase and *p*-cresol for 2 h, the films were removed from the reaction mixture and rinsed with deionized water. The chitosan film became colored by incubation in this reaction mixture, while the cellulose film remained clear. Consistent with this coloration, Fig. 1(a) shows that the reacted chitosan film had a substantial change in its UV–Vis absorbance. Since the only chemical difference in the repeating units of cellulose and chitosan is the C-2 amino group of chitosan, Fig. 1(a) suggests that these amino groups undergo reaction in the presence of tyrosinase and cresol.

The second approach to provide evidence that the enzymatic reaction results in a covalent modification of chitosan, involved the use of UV–Vis spectrophotometry for chitosan films treated in various ways. When chitosan films were incubated with buffered solutions containing either tyrosinase or *p*-cresol (but not both), Fig. 1(b) shows that the absorbance remained the same as that for the control film which had been incubated with only buffer. When a chitosan film was incubated with buffered solutions containing both tyrosinase and *p*-cresol, the film became reddish in color and after washing, the film remained red. Fig. 1(b) shows that this modified chitosan film had an absorbance peak at 305 nm and a broad shoulder around 360 nm relative to the unmodified chitosan film. Yamamoto et al. [32] who studied the enzymatic cross-linking of a synthetic tyrosine–lysine copolymer observed similar changes in the UV–Vis spectra after incubating this copolymer with tyrosinase. Although, they could not explain the increased absorbance at 305–310 nm, they proposed that the absorbance increase at 350–365 nm was due to the cross-linking reaction between the quinone (obtained by tyrosinase-catalyzed oxidation) and the amino group of the lysine residues. This group further proposed that the cross-linking involved a Michael-type addition.

The third approach to provide evidence that enzymatic reaction results in the covalent modification of chitosan was to monitor changes in the IR spectra of chitosan films which were incubated with tyrosinase and *p*-cresol for varying times. Because of the overlap between the hydroxyl and the amine stretching regions of the IR spectra and the abundance of hydroxyl groups in chitosan, IR cannot be used to obtain definitive proof that chitosan has been covalently modified at the amino group. Rather IR analysis provides

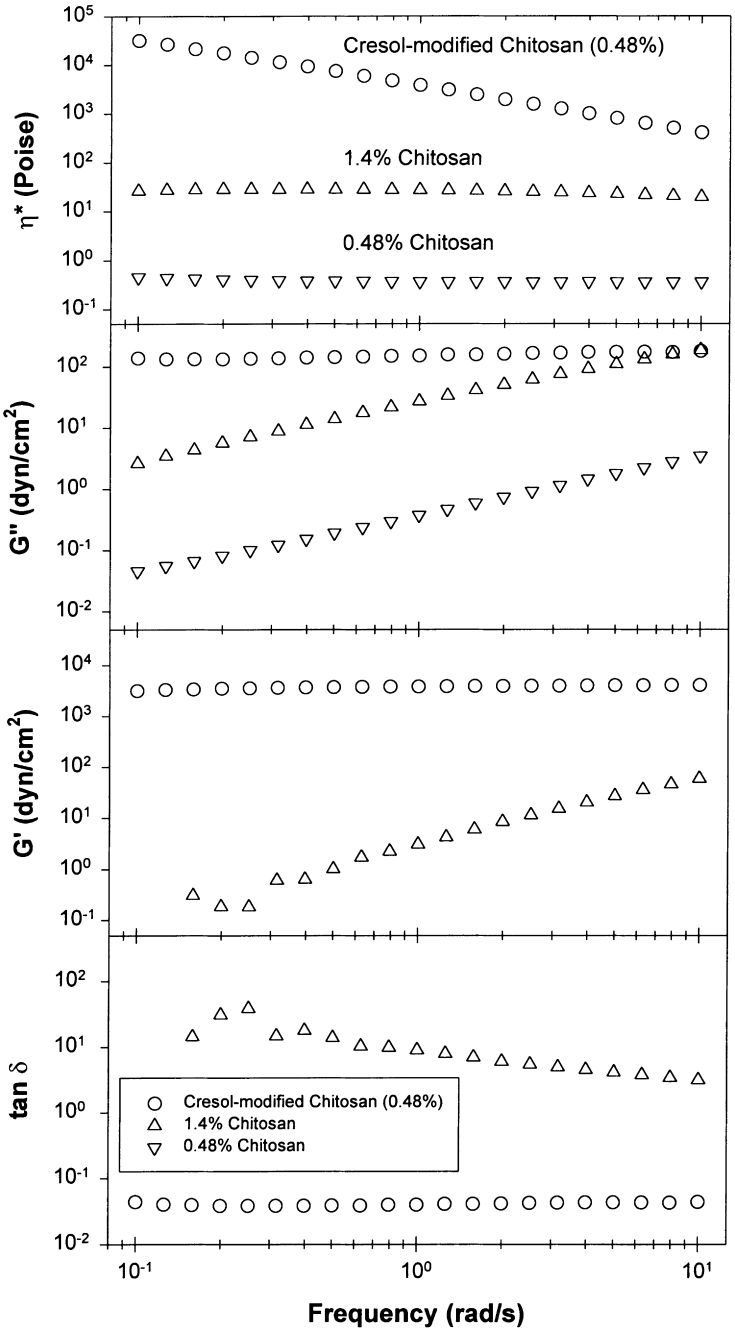


Fig. 5. Oscillatory shear measurements showing gel behavior of modified chitosan. Complex viscosity (η^*), viscous loss modulus (G''), storage modulus (G') and tan δ versus frequency (ω) for 0.48 and 1.4% chitosan solutions and for modified chitosan formed using 0.48% chitosan, 18 mM *p*-cresol (0.6 equivalents relative to chitosan amino groups) and 100 U ml^{-1} tyrosinase.

additional supporting evidence for such modifications. Specifically, Fig. 1(c) shows that during the course of enzymatic modification, there are progressive changes in both the NH stretching and NH bending regions of the IR spectra. In the NH stretching region (3200–3500 cm^{-1}), primary amines typically have two bands while secondary amines have a single band. Fig. 1(c) shows that during the course of the reaction there was a small decrease in the 3450 cm^{-1} peak which is consistent with the conversion of some primary amino groups of chitosan. Fig. 1(c) also shows the emergence of a peak in the NH bending region at 1590 cm^{-1} which may be associated with the formation of substituted amines and imines as suggested by Muzzarelli et al. [5].

In the final approach, we used ^{1}H NMR to analyze chitosan that had been modified under homogeneous conditions. For a homogeneous reaction, a 0.32% chitosan solution (20 mM equivalent amino groups assuming chitosan is fully deacetylated) at pH 6.0 was incubated with 30 mM *p*-cresol (1.5 equivalents of *p*-cresol to chitosan-N) and

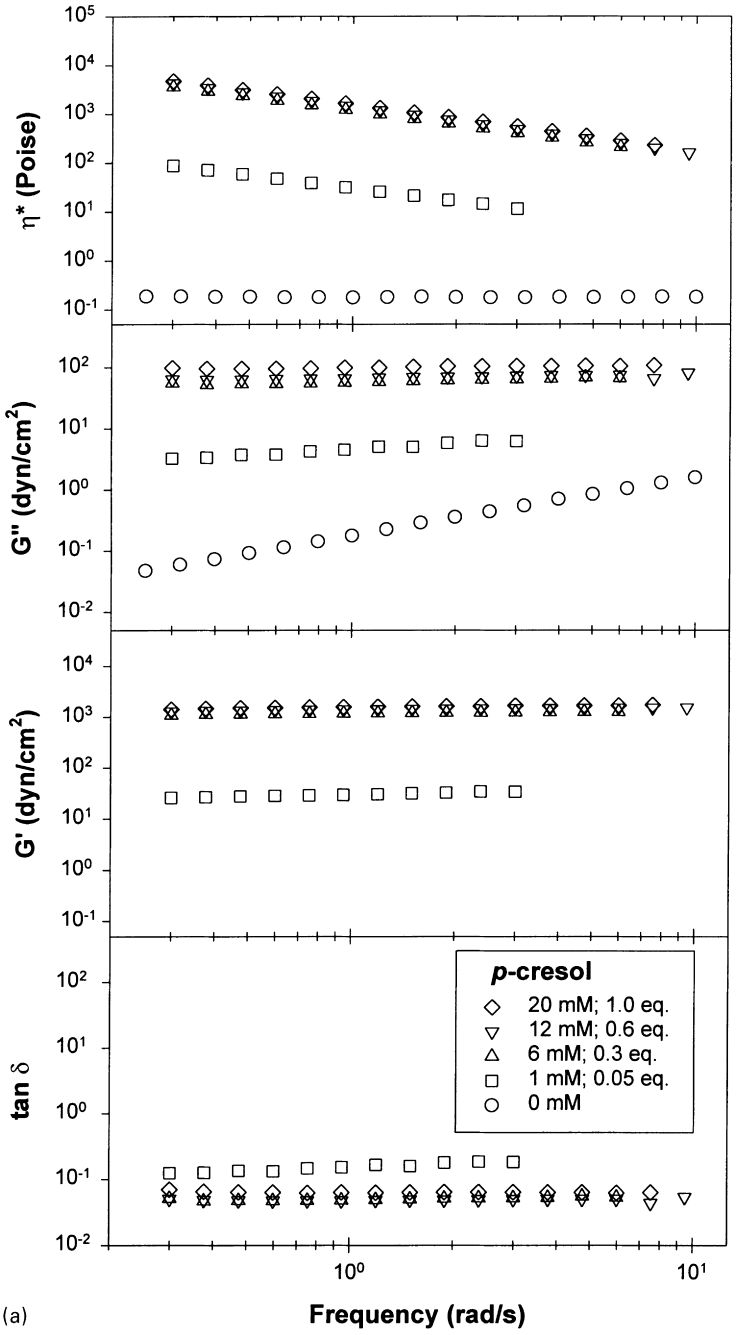


Fig. 6. The effect of varying concentrations on the rheological properties of modified chitosan gels. (a) Properties of modified chitosan (0.32%) formed with 1, 6, 12 or 20 mM *p*-cresol (0.05, 0.3, 0.6 or 1 equivalents relative to chitosan amino groups) and 20, 60, 80 or 100 U ml^{-1} tyrosinase. A control chitosan solution (0.32%) is designated as 0 mM. (b) Properties of modified chitosan formed from: 0.16, 0.32 or 0. 48% chitosan; 6, 12 or 18 mM *p*-cresol (0.6 equivalents relative to chitosan amino groups); and 60, 80, or 100 U ml^{-1} tyrosinase, respectively.

tyrosinase. During the reaction, the solution was converted into a viscous gel (see later). After reaction, the pH of the modified chitosan gel was adjusted to between 13 and 14, and stirred overnight. This procedure converts the gel into a solution in which the modified chitosan is dissolved. The ability of enzymatically-modified chitosan to dissolve at high pH was observed previously for chlorogenic-acid modified chitosan [16]. Presumably, the ability of cresol-modified chitosan to be solubilized above a pH of about eight involves deprotonation of the phenolic hydroxyl groups. Once the modified chitosan had been fully dissolved, the pH was reduced to seven where the modified polymer forms a brown precipitate. As described in Section 2, the modified chitosan was dissolved, precipitated and washed several times to remove any unconverted *p*-cresol or physically adsorbed phenolics.

After washing, the modified chitosan was exchanged with D_2O and dissolved under basic conditions and its ^1H NMR

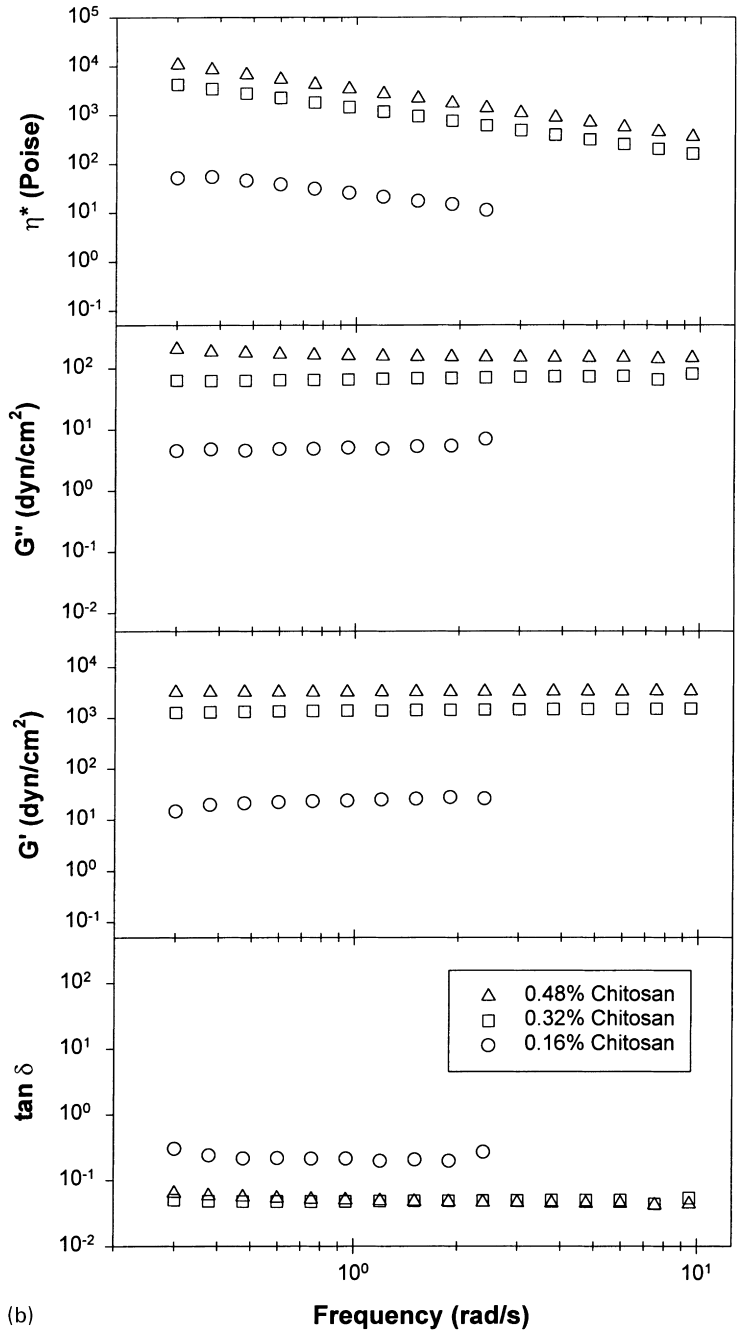


Fig. 6. (*continued*)

spectrum was compared to that of unmodified chitosan. Because unmodified chitosan is not base-soluble, its spectrum was obtained under acidic conditions. The characteristic peaks for the chitosan spectrum of Fig. 2(a) are 4.8 ppm for the anomeric proton on C-1, 3.1 ppm for the C-2 proton and 2.0 ppm for the methyl protons of the *N*-acetylglucosamine residues. Integration of the methyl peak indicates that about 8% of the sugar residues were acetylated. The peaks between 3.4 and 4.0 are for the protons on C-3, C-4, C-5 and C-6 [40]. Fig. 2(b) shows that the peaks for the cresol-modified chitosan are shifted upfield (i.e. the anomeric proton is shifted from 4.8 to 4.4 ppm, and the C-2 proton is shifted from 3.1 to 2.6 ppm), presumably, because the spectra was collected at a higher pH. Other than this upfield shift, the spectrum in Fig. 2(b) demonstrates that the base-soluble material is a derivative of chitosan. As observed previously [16], Fig. 2(b) shows only a weak downfield signal in the region expected for phenolic moieties suggesting that the modified chitosan has a low degree-of-substitution. Enlargement of the spectra for cresol-modified chitosan in the region for vinyl and aromatic protons (between 6.0 and 7.5 ppm) shows a

broad signal. Some signal broadening is expected when a moiety is grafted onto a polymer. However, the broadening observed between 6.0 and 7.5 ppm, may also be due to the modification by a heterogeneous mixture of phenolic moieties.

In summary, Figs. 1 and 2 provide evidence that chitosan undergoes covalent modification by the product(s) of the tyrosinase-catalyzed oxidation of *p*-cresol.

3.2. Steady shear viscosity measurements of enzymatically-modified chitosan solutions

To demonstrate that tyrosinase-catalyzed cresol oxidation results in changes in the rheological behavior of chitosan solutions, we monitored viscosity as a function of time for various solutions. In these experiments, we used solutions containing 0.32% chitosan (20 mM equivalent amine concentration assuming chitosan is fully deacetylated) at a pH of 6.0 and 12 mM *p*-cresol (0.6 equivalents relative to chitosan-N). As can be seen from Fig. 3, when chitosan solutions were incubated with both tyrosinase and *p*-cresol, the viscosity was observed to increase 2000-fold over a 10 h period. No viscosity increases were observed in control solutions that contained chitosan and *p*-cresol, chitosan and tyrosinase, or *p*-cresol and tyrosinase. The latter control indicates that chitosan is required for the viscosity to be enhanced and that oligomeric or polymeric phenols are not solely responsible for this behavior.

Fig. 4(a) shows that when 0.32% chitosan solutions were incubated with tyrosinase and varying levels of *p*-cresol, the viscosity increased sooner and to higher values as the *p*-cresol concentration was increased. Presumably, higher initial cresol concentrations resulted in more rapid quinone formation and possibly a more rapid cross linking of chitosan. Similar observations were made by Roberts and Taylor [7] who reported the gelation time for glutaraldehyde-cross-linked chitosan to decrease with increasing glutaraldehye levels. For Pt(II) cross-linked chitosan gels, Brack et al. [41] reported decreased gelation times with increasing Pt(II) levels. Also, Moore and Roberts [9], and Vachoud et al. [35] reported that increases in the acetic anhydride levels resulted in earlier onsets for the formation of physical chitosan gels.

Fig. 4(b) shows that the viscosity of cresol-modified chitosan increases substantially with increasing chitosan concentration. It should be noted that the *p*-cresol levels in Fig. 4(b) corresponds to 0.6 mol *p*-cresol per mole of chitosan amino groups, and thus the *p*-cresol concentration was increased along with the chitosan concentration. It should also be noted that because of its high viscosity, it was not possible to obtain steady shear measurements for the 0.48% chitosan solution after 24 h of reaction.

3.3. Oscillatory shear measurements of enzymatically-modified chitosan solutions

Fig. 5 compares the rheological properties of chitosan solutions with those for a cresol-modified chitosan gel. For the 0.48 and 1.4% chitosan solutions, the complex viscosity (η^*) is independent of frequency indicating that these samples behave as Newtonian fluids. Fig. 5 shows that η^* for the cresol-modified chitosan is considerably larger than those for the chitosan solutions and η^* decreases with frequency which is characteristic of viscoelastic systems.

Storage (G') and loss (G'') moduli and the loss tangent ($\tan\delta = G''/G'$) are shown in Fig. 5 for the 1.4% chitosan solution and for a modified chitosan gel formed from 0.48% chitosan. Data for G' and $\tan\delta$ for the unmodified 0.48% chitosan solution are not shown because G' values were too low to be accurately measured. As shown in Fig. 5, G' for the 1.4% chitosan solution increases with frequency and the $\tan\delta$ is significantly greater than one, further indicating that this sample behaves as a liquid. In contrast, the *p*-cresol-modified chitosan behaves as a gel, in that G' is independent of frequency and $\tan\delta$ is significantly less than one [42,43]. Dynamic oscillatory shear studies showed substantial increases in G' and G'' for the glutaraldehyde cross-linked [38] and ionically cross-linked chitosans [44]. In both cases, the $\tan\delta$ for these gels was less than 0.1. In contrast, Delben et al. [45] reported that 2% solutions of N-(carboxymethyl)-chitosan showed considerable pseudo-plastic behavior (η^* decreased with ω) with storage and loss moduli of the same order of magnitude although $\tan\delta$ exceeded one. They concluded that this weak gel behavior of *N*-(carboxymethyl) chitosan may be due to weak network-forming associations.

Fig. 6 shows results for gels formed with differing levels of cresol and chitosan. Fig. 6(a) shows that the use of *p*-cresol levels of only 1 mM (0.05 equivalents relative to chitosan-N), results in a significant increase in η^*, substantial pseudo-plastic behavior (i.e. η^* decreases with increasing ω), and storage and loss moduli which are nearly independent of ω. Above about 6 mM (0.3 equivalents relative to chitosan-N), increasing *p*-cresol levels have a lesser effect on the gel's rheological properties. Fig. 6(b) shows that increases in chitosan levels substantially increase η^* and the gel's strength (as measured by G').

Although it would be desirable to relate the rheological behavior to the underlying gelation chemistry [41], the complexity of the quinone reactions precludes definitive characterization. However, two lines of reasoning suggest that a covalent cross linking mechanism is involved in gel formation. First, there are considerable similarities between our system and other better-studied systems involving natural and synthetic polymers containing tyrosine (or DOPA) and lysine residues. It is known that these peptides can be oxidized by tyrosinase and that the resulting quinones can undergo covalent cross-linking reactions with the amino groups [24,26,27,32]. Yamamoto [32] and Yu and Deming [27] also reported increased viscosities after incubation of such peptides with tyrosinase. Second, enzymatic modification led to large changes in the rheological properties even when chitosan concentrations were considerably less than 1%. These concentrations are in the

lower semi-dilute concentration region. In contrast, when physical intermolecular associations are exploited to enhance viscosities, it appears that higher chitosan concentrations are required. Specifically, Kjoniksen et al. [36,37] reported that the hydrophobically-modified chitosans showed dramatic increases in η^* only when the polymer concentration exceeded about 1% (in the absence of surfactants). Although a covalent cross-linking mechanism appears likely for cresol-modified chitosan gels it does not preclude the possible involvement of alternative, physical mechanisms for junction formation. Further, a covalent cross-linking mechanism may be somewhat reversible in this system, since Schiff bases (which are possible linkages) can be reversed in aqueous environments. In fact, over the course of weeks, we have observed the loss of gel structure and reductions in steady shear viscosity of cresol-modified chitosan.

3.4. Biological degradation of cresol-modified chitosan gels

To suggest whether *p*-cresol-modified chitosan would be biodegradable, we incubated this gel with a commercially available chitosanase known to hydrolyze chitosan's backbone [46]. Fig. 7 shows that the viscosity of the *p*-cresol-modified chitosan gel was rapidly lost by incubation with chitosanase indicating that this modification does not prevent its biological degradation. Muzzarelli et al. [30] also observed that chitosan cross-linked with similar phenolic moieties were susceptible to various hydrolytic enzymes. These observations of the biological degradation of modified chitosan can be contrasted with observations that modification of cellulose by bulky substituents can disrupt its biodegradability [47].

4. Conclusion

Chitosan is an interesting polymer because it can be obtained from abundant and renewable resources, and because it has a high content of primary amino groups. The basicity of these amino groups allows chitosan to be homogeneously modified in aqueous environments while the nucleophilicity of these groups allows modification under facile conditions. In this work, chitosan was modified using the enzyme tyrosinase which converts a low molecular weight phenolic compound into a reactive quinone that diffuses from the enzyme's active site and reacts with the polymer. This approach to polymer modification is simple and rapid compared to alternative enzymatic approaches (e.g. trans-esterification approaches) which require binding of both the polymer and the substituent moiety at the enzyme's catalytic site [48-52]. The disadvantage of using tyrosinase is that the quinone reactions can be difficult to control and the modified chitosan may consist of an array of monomeric and oligomeric phenolic substituents.

The results from this study demonstrate that tyrosinase can be used to modify the chemical and physical properties of chitosan. Chemical evidence for covalent modification was provided by UV–Vis and IR spectra of heterogeneously-modified chitosan films (Fig. 1), and ^{1}H NMR analysis of homogeneously-modified chitosan solutions (Fig. 2). These results suggest that the reaction involves chitosan's amino groups. Due to the diversity of the possible quinone reactions, and the low degree of modification, it was not possible to identify specific linkages between the quinoids and chitosan. The physical properties of a semi-dilute chitosan solution were substantially altered by incubation with tyrosinase and *p*-cresol. Specifically, the steady shear viscosity increased dramatically during incubation (Fig. 3), and oscillatory shear measurements indicated that the chitosan solution was converted into a gel (Fig. 5). Again, the complexity of the system precludes a definitive characterization of the gelation mechanism. As indicated in Scheme 2, the quinone generated by tyrosinase is multifunctional and the possibility exists, that gelation results from a chemical cross-linking of chitosan chains. Finally, the ability of chitosanase enzymes to rapidly destroy the gel (Fig. 7) indicates that the enzymatically-modified chitosan remains biodegradable.

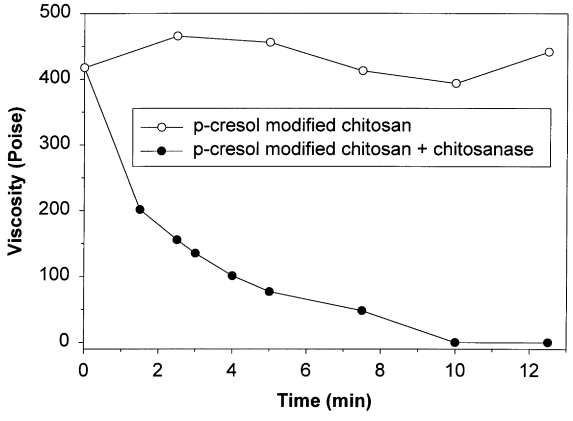


Fig. 7. Biodegradation of modified chitosan by the enzyme chitosanase. The modified chitosan was prepared 0.32% chitosan, 12 mM *p*-cresol and 80 U ml^{-1} tyrosinase and these gels were incubated in the presence and absence of chitosanase (1 U in 15 ml of gel).

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