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Enzyme-catalysed polymer modification: reaction of phenolic compounds with chitosan films

Gregory F. Payne* **and Mahesh V. Chaubal**
Department of Chemical and Biochemical Engineering and Center for Agricultural Biotechnology, University of Maryland, Baltimore County, 5401 Wilkens Ave., Baltimore, MD 21228, USA

and Timothy A. Barbari
Department of Chemical Engineering, The Johns Hopkins University, 3400 North Charles St, Baltimore, MD 21218-2694, USA
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One method of synthesizing new materials is to react functional pendant groups on an existing polymer. Here, an enzymatic strategy was investigated as a method of producing new polymers from chitosan. The enzyme used was tyrosinase which is known to oxidize a range of phenolic reactants, yielding reactive *o*-quinones. The products of this enzymatic oxidation appear to react with, and alter the chemical properties of chitosan. Specifically, it was observed that when thin chitosan films were treated with both tyrosinase and a phenolic reactant, the u.v.–visible spectra and the acid–base properties were markedly altered compared with films that were untreated. Copyright © 1996 Elsevier Science Ltd.

(Keywords: chitosan; enzyme; tyrosinase)

Introduction

Synthetic polymers have become an integral part of modern society and serve a variety of functions ranging from structural materials in automobiles to soft contact lenses. Despite their widespread use, synthetic polymers and polymer processes are being re-evaluated for a variety of reasons. First, the synthetic steps required to produce traditional polymers are often hazardous and environmentally unfriendly. A second concern is that a nonrenewable resource (i.e. petroleum) is used to produce the majority of polymers. Finally, the inertness of most synthetic polymers has caused considerable concern given that 20% of the input to landfills consists of polymers[1]. This concern has stimulated the development of recyclable and biodegradable polymers[2,3]. For the above reasons there is a growing interest in developing biodegradable polymers that use renewable resources and environmentally benign synthetic steps.

Several biological approaches have been considered for the development of natural and biodegradable polymers. For instance, fermentation processes are used to produce the copolymers of hydroxybutyrate and hydroxyvalerate[4,5] which are used in moulded containers. A second approach to biopolymers is to use living systems to produce monomers which are then synthetically polymerized. For example, lactic acid, which can be produced by fermentation, is used in the synthesis of the lactic–glycolic acid copolymers which are used in a variety of medical applications[6]; acrylamide is produced in Japan by the bioconversion of acrylonitrile[7].

A third biological route currently being researched is the use of enzymes to produce polymers[8]. A common enzyme-catalysed polymerization approach is to use hydrolytic enzymes in nonaqueous environments such that reverse, polycondensation, reactions are catalysed. Typical enzymes include lipases[9–11], hydrolases[12] and proteases[13] to form polyesters or polyamides. Problems remaining with this approach are that non-aqueous solvents are required, the reactions can be slow (taking days) and products generally have only modest molecular weights (e.g. 30 000). An alternative enzyme-catalysed polymerization approach is to use enzymes, such as horseradish peroxidase, which catalyse free radical polymerization reactions[14,15].

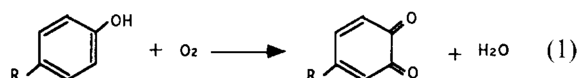
Reactions of natural and synthetic polymers to produce new materials is an alternative to conventional polymerization. For instance, the commonly used cellulosic polymers, carboxymethylcellulose, cellulose acetate, and nitrocellulose, are derived through the chemical modification of cellulose. Unfortunately, rather harsh conditions are required to functionalize cellulose: the chlorinated reagent monochloroacetic acid is used to form carboxymethylcellulose, strong acids are required for producing cellulose acetate, and explosive conditions are approached during the nitration of cellulose to form nitrocellulose.

In the work reported here, the natural polymer chitosan was studied as an alternative to cellulose for chemical modification. The primary amine group of the repeating glucosamine unit confers electrolyte and nucleophilic properties to the chitosan polymer. Despite the industrial interest in polyamines, there are few amine-rich natural polymers while synthesis of amine-containing polymers generally requires relatively hazardous reactions (e.g. chloromethylation of the polymer followed by amine addition). As a result, recent research efforts have examined how to tailor

* To whom correspondence should be addressed

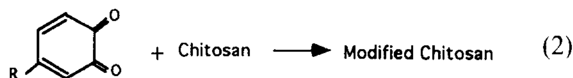
the properties of chitosan through crosslinking, functionalization, copolymerization and blending[16–25]. Our goal was to determine whether an enzymatic approach could simply and cleanly modify chitosan.

For polymer modification, we examined the enzyme tyrosinase which catalyses the reaction of phenols to *o*-quinones. *o*-Quinones are considerably more reactive than phenols and, based on previous work[26–31], it seemed reasonable that the *o*-quinones would react with chitosan. Specifically, reaction (1) shows the tyrosinase-catalysed oxidization of phenols to *o*-quinones[32–34]. Because tyrosinase uses molecular oxygen as the oxidant, neither complex cofactors nor cofactor regeneration are required in this enzymatic oxidation step.



(1)

In reaction (2), the nucleophilic amine functionality of chitosan is exploited for reaction with the unstable quinone.



(2)

In earlier studies, we observed that quinones bind to chitosan with an enthalpy of $-25\,kcal\,mol^{-1}$ [26] which is consistent with the involvement of strong, covalent interactions. In contrast to quinones, phenols were observed to be unable to bind to chitosan and thus the quinones appear to undergo a specific chemical reaction with chitosan and this reaction presumably modifies the chitosan polymer. An extensive body of literature on the binding of amines to enzyme-activated phenolic soil components indicates that the chemistry of binding can involve a variety of mechanisms[35–39]. In other studies, polymerization reactions[40] and polymer modification reactions[41] with amines have been reported to involve Michael's-type additions.

The specific goal of this study was to demonstrate that this enzymatic approach can chemically alter chitosan gel films.

Materials and methods

Chitosan, catechol and mushroom tyrosinase were obtained from Sigma Chemical Co. The tyrosinase was reported by the manufacturer to have a specific activity of $2400\,U\,mg^{-1}$. Phenol, 4-tert-butylcatechol and *p*-hydroxyphenoxyacetic acid were obtained from J. T. Baker, Lancaster, and Pflatz and Bauer, respectively. All other chemicals were obtained from Fisher Scientific.

Chitosan was dissolved by adding 2 g chitosan to 100 ml of an aqueous solution containing 5 v/v% acetic acid. The viscous chitosan solution was stirred overnight. Chitosan films were made by spreading 0.6 g of the chitosan solution (11.4 mg dry chitosan) onto glass microscope slides. The chitosan was gelled by immersion for 1 h into 25 ml of a 1 M NaOH solution. After gelation, the films were washed thoroughly with distilled water and air dried at room temperature. Unless otherwise noted, the films remained adhered to the microscope slide throughout the studies. For modification, the air-dried films were added to 25 ml of a 50 mM potassium phosphate buffer (pH 7.2) containing the phenolic reactant and $48\,U\,ml^{-1}$ tyrosinase ($20\,mg\,l^{-1}$).

For reaction of chitosan flakes, 2 g flakes were incubated overnight in 200 ml of buffer (50 mM potassium phosphate, pH 7.2) containing $48\,U\,ml^{-1}$ tyrosinase and 60 mM of various phenols. The phenol level in this experiment was chosen to be equivalent to the theoretical capacity of chitosan assuming all the phenol was converted and bound to a single amine site, and the chitosan was fully de-acetylated. After reaction, the chitosan flakes were then recovered by sieving and suspended in 100 ml of an aqueous solution of 0.1 M HCl. This 2–3 h acid treatment was used to dissolve unconverted chitosan. After this dissolution step, the reacted chitosan flakes were recovered by sieving through a 200 mesh stainless steel screen and then thoroughly washed with distilled water. After washing, the modified chitosan flakes were dried at room temperature for approximately 1 day.

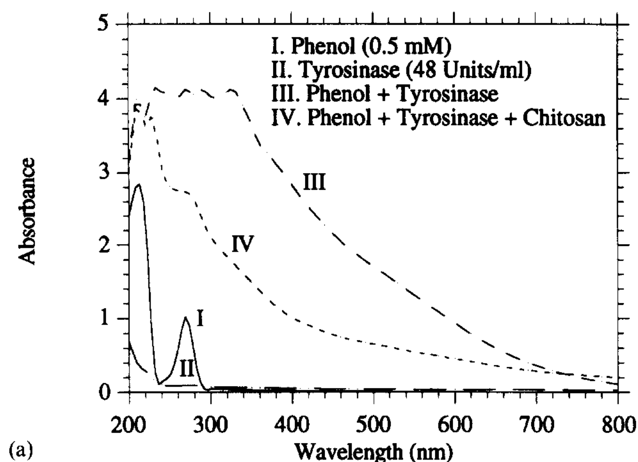
Ultraviolet (u.v.)–visible spectrophotometry was conducted using a diode array spectrometer (Hewlett–Packard Model 8452A). To obtain spectra for modified chitosan films, the glass slides were placed in the spectrophotometer perpendicular to the light-path such that the light passed directly through the film.

The pH was measured by a pH electrode (Accumet Model 915).

Results and discussion

To provide spectroscopic evidence that chitosan can be enzymatically modified, we contacted chitosan films with aqueous solutions containing various components. For comparison, *Figure 1a* shows the u.v.–visible spectra for the liquid phase after each incubation. Curve I in *Figure 1a* shows the typical spectra for a 0.5 mM solution of phenol, while Curve II shows the spectra for a solution containing $48\,U\,ml^{-1}$ of mushroom tyrosinase ($20\,mg\,l^{-1}$ protein). Curve III shows the results when a solution containing both phenol and tyrosinase was incubated overnight; as can be seen from this curve, the absorbance increased markedly and the peak was broadened, which is consistent with the hypothesis that phenol is converted into the quinone and the quinones are subsequently polymerized into oligomeric phenols. When a chitosan film was initially added to a solution of phenol (0.5 mM) and tyrosinase, Curve IV shows a reduction in absorbance compared to Curve III. This reduction is consistent with the hypothesis that some of the quinone formed from the tyrosinase-catalysed reaction of phenol is removed from the solution by binding to chitosan.

Figure 1b shows the u.v.–visible spectra for the chitosan film compared with the same film prior to treatment (i.e. the curves in *Figure 1b* are difference spectra of the chitosan films). Curve I of *Figure 1b* shows that when the absorbance of the chitosan film was measured again there was a low level of 'noise' at small wavelengths (i.e. less than 260 nm). When the film was incubated overnight with a solution of phenol (0.5 mM) and then thoroughly washed with distilled water, Curve II of *Figure 1b* shows that the u.v. absorbance was not substantially altered compared with Curve I. When a



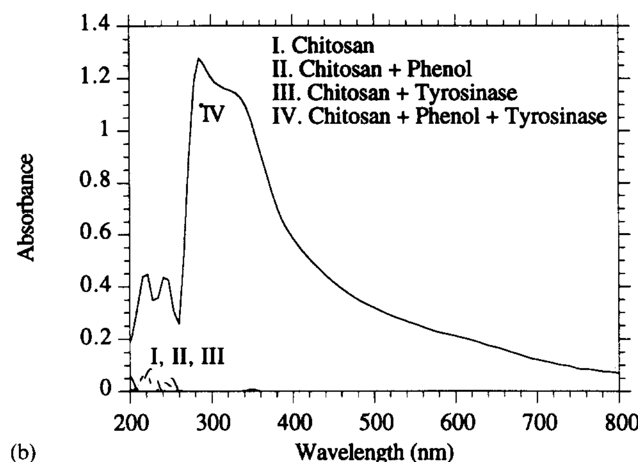


Figure 1 Enzymatic modification of chitosan films. (a) U.v.–visible spectra of buffered solutions containing: phenol (Curve I); mushroom tyrosinase (Curve II); phenol plus tyrosinase (Curve III); and phenol, tyrosinase and chitosan film (Curve IV). (b) U.v.–visible difference spectra of chitosan films after incubation with: buffer alone (Curve I); a buffered phenol solution (Curve II); a buffered tyrosinase solution (Curve III); and a buffered solution containing both phenol and tyrosinase (Curve IV). The films were prepared on glass slides and consisted of 11.4 mg chitosan on a dry weight basis. The incubation solution was 25 ml of 50 mM potassium phosphate buffer (pH 7.2). When used, the phenol concentration was 0.5 mM and the tyrosinase activity was 48 U ml^{-1}. The films were incubated overnight and washed extensively with distilled water prior to measuring the spectra

solution containing tyrosinase (but not phenol) was incubated with the chitosan film, Curve III showed very little change in absorbance compared with the control (Curve I). Finally, when both tyrosinase and phenol were incubated with the chitosan film, the film was observed to change from a whitish to a brownish colour. After washing with distilled water, the brown colour was retained in the film. When the spectra for this film was measured (Curve IV), the absorbance was observed to be substantially increased. It should be noted that the chitosan film used in *Figure 1b* is the same film used in Curve IV of *Figure 1a*. Thus, the results in *Figure 1* support the conclusion that tyrosinase is capable of catalysing the conversion of phenol to quinone and this quinone can then be covalently bound to the chitosan gel. In contrast, phenol by itself cannot be bound to chitosan (i.e. Curve II of *Figure 1b*).

To further support the above conclusions, we contacted a series of chitosan films with solutions containing tyrosinase and various concentrations of phenol. After

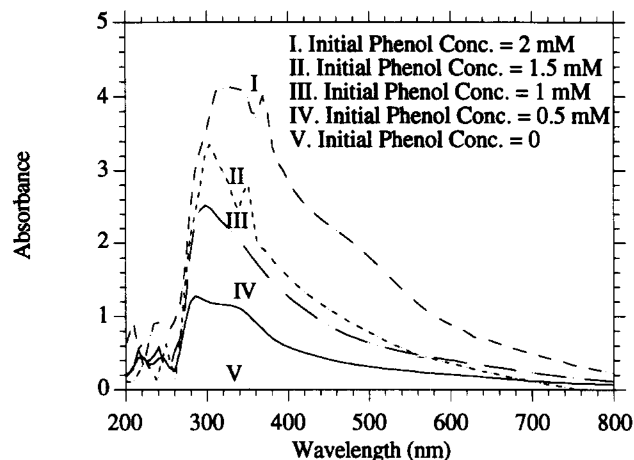


Figure 2 Chitosan films modified with varying amounts of phenol. Chitosan films (11.4 mg on a dry weight basis) were incubated with 25 ml buffered solutions (50 mM potassium phosphate, pH 7.2) containing 48 U ml^{-1} tyrosinase and various levels of phenol. The films were incubated for 6–8 h after which they were washed with distilled water. The spectra represent the difference between the modified film and the film prior to reaction

incubation overnight and washing with distilled water, the u.v.–visible spectra of the various films were measured and referenced against the absorbance of the same film prior to reaction (i.e. difference spectra were measured). As shown in *Figure 2*, the absorbance of the chitosan films increased in a nearly proportional manner with the level of phenol used for modification. It should be noted that the phenol levels in the experiment were selected to be approximately 20–80% of the theoretical capacity of the chitosan assuming: (1) that the chitin had been completely de-acetylated; (2) that all the added phenol was oxidized by the enzyme; and (3) that each product of the phenol oxidation binds to a single amine site of chitosan. These assumptions were made simply to calculate an appropriate phenol concentration range and are not likely to be entirely valid. For instance, the u.v.–visible absorbance of the solutions (data not shown) indicated that not all the phenol added to the solutions was bound to the chitosan films.

To test the generality of our observations, we examined three additional phenolic reactants. As shown in *Figure 3*, when chitosan films were contacted with solutions containing tyrosinase and various phenols, the films exhibited significant u.v.–visible absorbance. Again it should be noted that the spectra in *Figure 3* are difference spectra in which the absorbance prior to reaction is subtracted from the absorbance after reaction. As can be seen from *Figure 3*, all the chitosan films were chemically altered by incubation with tyrosinase and the *para*-substituted phenols tested.

In addition to demonstrating chemical changes using u.v.–visible spectrophotometry, we wished to demonstrate that the physicochemical properties of chitosan were also altered by the enzymatic reaction. Initially, we reacted chitosan flakes and measured changes in the acid–base behaviour[42,43]. After incubating chitosan flakes with tyrosinase and various phenols the flakes were observed to have turned dark brown, indicating that the polymer had been modified. After recovering the chitosan flakes, they were suspended in 0.1 M HCl to dissolve any unconverted chitosan. Finally, the modified chitosan flakes were recovered by sieving, washed

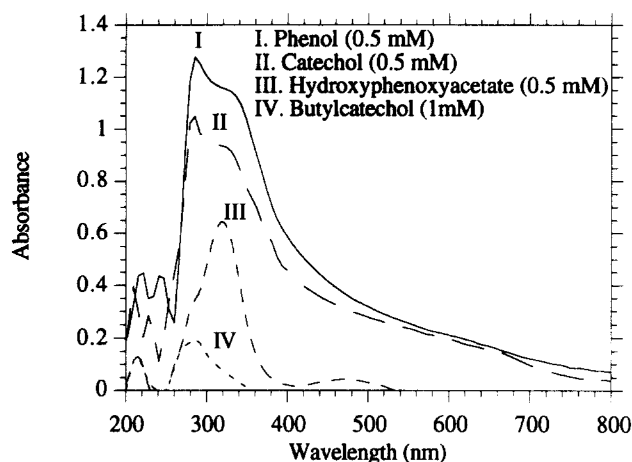


Figure 3 Chitosan films modified with various phenols. Chitosan films (11.4 mg on a dry weight basis) were incubated with 25 ml buffered solutions (50 mM potassium phosphate, pH 7.2) containing 48 U ml^{-1} tyrosinase and various phenolic compounds. The films were incubated for 6–8 h after which they were washed with distilled water. The spectra represent the difference between the modified film and the film prior to reaction

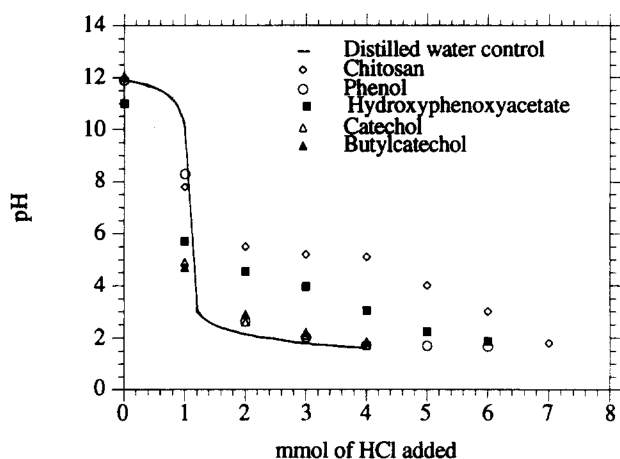


Figure 4 Acid–base titration curves for chitosan flakes modified with different types of phenols. For reaction, chitosan flakes (2 g on a dry weight basis) were incubated in 200 ml of buffer (50 mM potassium phosphate, pH 7.2) containing 48 U ml^{-1} tyrosinase and 60 mM of various phenols. After incubating overnight, the flakes were recovered by sieving, added to 100 ml of 0.1 M HCl to dissolve away any unreacted chitosan, and then washed extensively with distilled water and air dried. For titration, 1 g of dried flakes were added to 100 ml of distilled water containing 1 mmol NaOH and incremental additions of 1 mmol HCl were made. Except for the first, high pH reading, all samples were allowed to equilibrate for at least 3 h before the pH was measured and subsequent acid additions were made

thoroughly with distilled water and air dried at room temperature.

To demonstrate changes in the acid–base behaviour of the modified chitosan flakes, titration curves were measured. For comparison, the titration of the distilled water control is shown as the solid curve in *Figure 4*. In this control, 1 mmol NaOH was initially added to 100 ml of distilled water, and the pH was recorded as a function of subsequent HCl additions. As can be seen from the control in *Figure 4*, the addition of 1 mmol HCl results in the neutralization of the base, while further additions of HCl result in marked pH reductions. *Figure 4* also shows the titration curve for 1 g of dry, unmodified chitosan flakes which has been added to 100 ml of distilled water containing 1 mmol NaOH. As acid was added, the chitosan flakes were observed to dissolve. *Figure 4* suggests the pK_a for chitosan to be between 4 and 6, which is similar although somewhat lower than the value of 6.3 used by other authors[44,45]. Also, *Figure 4* shows a capacity of approximately 5.2 meq g^{-1} for the chitosan flakes which compares to the theoretical value, assuming complete de-acetylation of chitin, of 6.1 meq g^{-1}.

To perform titration curves for modified chitosan flakes, 1 g of modified flakes was added to 100 ml of distilled water containing 1 mmol NaOH. As initial studies indicated that incubation of modified chitosan under basic conditions results in the release of u.v.-absorbing material into the solution, the pH of the initial solution was measured and 1 mmol HCl was immediately added. Thus the initial, high pH reading does not represent an equilibrium measurement. After each addition of HCl, the suspensions were equilibrated for at least 3 h before the pH was measured and a subsequent acid addition was made. When chitosan was modified by reaction with neutral phenols (phenol, catechol and tert-butylcatechol), *Figure 4* shows the titration curves were similar to the titration curves for the distilled water control. This observation is consistent with the hypothesis that the modification reaction consumes the amine groups of chitosan and forms a product which lacks the ability to associate with the hydrogen ion in the pH range studied.

Figure 4 shows also the titration curve for 1 g of chitosan flakes reacted with the acidic phenol, *p*-hydroxyphenoxyacetic acid. As can be seen, the titration curve for this modified material indicates that an ionizable moiety is present which titrates at a pH of about 4—presumably this is the carboxylate moiety of the phenoxyacetate group. The capacity for these modified chitosan flakes was observed to be 3.0 meq g^{-1}. A theoretical capacity of 2.9 meq g^{-1} can be calculated assuming all the nitrogen groups of chitosan react as a Michael's adduct with the *o*-quinone of hydroxyphenoxyacetate (i.e., a molecular weight of 345 for the repeating unit of the modified polymer).

To confirm that modification alters the acid–base behaviour of chitosan films, we reacted chitosan films by incubation with tyrosinase and various phenols (assuming complete de-acetylation of chitin, the amount of phenol used corresponded to twice the capacity of the chitosan). *p*-Hydroxyphenoxyacetic acid-modified films could not be studied because extensive reaction with this acidic phenol resulted in films that were not retained on the microscope slide but became suspended in the incubation solution. After modification, the films were washed with distilled water and air dried. To titrate the modified films, they were immersed in 10 ml of distilled water containing 0.01 mmol NaOH. To limit the release of reacted groups from chitosan, the pH was measured after immersing the films in the caustic solution and immediately 0.01 mmol HCl was added to the solution. Thus, the first, high pH measurement in *Figure 5* does not reflect an equilibrated film. After each 0.01 mmol acid addition, the samples were equilibrated for at least 3 h before measuring the pH. As can be seen from *Figure 5*, the titration curve for the chitosan film indicates a broad pK_a in the region of 4.5 to 7. The capacity of the chitosan film was observed to be 5.5 meq g^{-1} which compares to the theoretical value

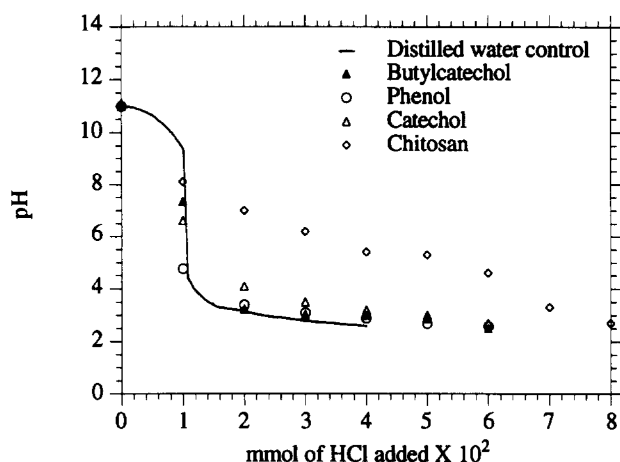


Figure 5 Acid–base titration curves for chitosan films modified with different types of phenols. Chitosan films (11.4 mg on a dry basis) were modified by incubation for 6–8 h in 25 ml buffered solutions (50 mM potassium phosphate, pH 7.2) containing 48 U ml^{-1} tyrosinase and 5 mM of various phenols. After incubation, the films were washed extensively with distilled water and air dried. For titration, each film was added to 10 ml of distilled water containing 0.01 mmol NaOH and incremental additions of 0.01 mmol HCl were made. Except for the first, high pH reading, all samples were allowed to equilibrate for at least 3 h before the pH was measured and subsequent acid additions were made

of 6.1 meq g^{-1}, assuming completely de-acetylated chitin.

When the chitosan films were reacted with the neutral phenols (phenol, catechol, and tert-butylcatechol), the titration curves were observed to be similar to that for the distilled water control. This similarity indicates that the modified chitosan films did not contain a significant number of groups which could be protonated in the pH range studied. These results are consistent with results from *Figure 4* and support the hypothesis that the amine functional groups are consumed by the enzymatically generated quinones.

Conclusions

Polymers are used in a wide range of applications, with the synthesis, crosslinking or modification reactions being tailored to confer the necessary physical, chemical and mechanical properties to the polymer. Often, these reactions start with nonrenewable resources and employ harsh processing conditions, while the resulting polymeric products are generally nonbiodegradable or only slowly degraded in the environment. Chitosan is derived from renewable resources and the primary amine groups on the polymer can be readily reacted under a range of conditions. In this work, we have demonstrated that the enzyme tyrosinase can convert phenols to products (i.e. *o*-quinones) which react with, and alter the chemistry of, chitosan films. There are several reasons why we believe this enzymatic modification approach is potentially important.

First, the enzymatic oxidation functions as an 'activating' reaction, such that an otherwise unreactive phenol is converted into a reactive *o*-quinone. This *in situ* activation suggests potential for improved safety compared with the direct use of reactive raw materials. Present methods for polymer modification through reaction involve either strong acids (e.g. for nitration and sulfonation) or reactive reagents (e.g. chloromethyl methyl ether, ethylene oxide, or acid chlorides). If relatively unreactive reagents can be 'activated' *in situ*, the health and safety concerns for storage and handling are greatly reduced. Also advantageous is the broad substrate specificity of tyrosinase which permits the enzymatic 'activation' reaction to be performed with a variety of phenolic reactants.

Second, this enzymatic route to polymer modification is operationally simple and environmentally friendly. Because both the enzyme-catalysed and chitosan-binding reactions occur in aqueous solution (assuming the phenolic reactant is soluble), anhydrous solvents are not required; since the reaction occurs under ambient conditions, it is possible to consider modifying chitosan with labile phenolic compounds (e.g. peptides). In addition, the enzymatic reaction is rapid, simple and requires no special incubation procedures or temperature ramping.

Finally, chitosan is a readily available natural resource and it seems reasonable to speculate that modified chitosan products could be biodegradable. Obviously, however, biodegradability would need to be demonstrated experimentally.

Although this work suggests the potential for enzymatically modifying chitosan, there are several issues which remain to be resolved. Although chitin and chitosan can be obtained from renewable wastes (crab, shrimp and lobster shells), current processes for chitin recovery require acid and base treatments to remove the calcium carbonate and protein, respectively. For chitosan production, the chitin is typically de-acetylated using a strong base. Because of the current scale, production is relatively inefficient and results in chitosan that is rather expensive. More efficient manufacturing operations and the potential for manufacturing steps that have reduced requirements for acid and base (e.g. enzymatic deproteination and deacetylation) could make chitosan manufacturing less expensive and more environmentally benign. To be commercially exploited, the enzymatically modified chitosan must also have appropriate physical, chemical or mechanical properties for a particular application. Although this study demonstrates that chitosan films can be chemically altered by enzymatic reaction, the structural details of the modification, and the resulting changes in physical and mechanical properties of these polymers need to be further characterized.

Acknowledgements

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