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Enzymatic Grafting of Peptides from Casein Hydrolysate to Chitosan. Potential for Value-Added Byproducts from Food-Processing Wastes

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Tyrosinase was used to initiate the grafting of peptides onto the amine-containing polysaccharide chitosan. Chemical evidence for covalent grafting was obtained from electrospray mass spectrometry for products formed from reactions with glucosamine (the monomeric unit of chitosan) and the model dipeptide Tyr-Ala. When this model dipeptide was incubated with tyrosinase and chitosan, there was a marked increase in the viscosity of the solution. This viscosity increase provides physical evidence that tyrosinase can initiate peptide grafting onto the chitosan backbone. A peptide-modified chitosan derivative was generated by reacting chitosan (0.32 w/v%) with acid-hydrolyzed casein (0.5 w/v %) using tyrosinase. After reaction, the peptide-modified chitosan was partially purified and dissolved in an aqueous acetic acid solution. Low concentrations of this peptide-modified chitosan were observed to confer viscoelastic properties to the solutions. Specifically they conferred high viscosities and shear thinning properties to the solutions, and solutions containing only 1 w/w % of the peptide-modified chitosan behaved as weak gels. Thus, tyrosinase provides a simple and safe way to convert food-processing byproducts into environmentally friendly products that offer useful functional properties. The selectivity of tyrosinase and the relatively high reactivity of chitosan's amines allow grafting to be performed with uncharacterized peptide mixtures present in crude hydrolysates.

KEYWORDS: Biobased products; casein hydrolysate; chitosan; enzymes; peptides; gelatin; renewable resources; sustainability; tyrosinase; viscoelasticity

INTRODUCTION

There is a growing environmental concern that agricultural and food-processing wastes are contributing excessive nutrient burdens to local ecosystems (*1*−*3*). The conventional approach to lessening such burdens is to institute better waste-management practices (e.g. composting or activated sludge treatments). These concerns are providing a renewed incentive for generating value-added byproducts. Why incur waste-management costs if investments in manufacturing could yield salable byproducts? We are examining how byproducts from food-processing wastes can be converted into value-added functional polymers. Specifically, we exploit an enzymatic method to graft side groups and side chains onto a polysaccharide backbone to confer useful viscoelastic properties.

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The polysaccharide for our study is chitosan that is obtained by the deacetylation of chitin. Chitin is found in crustaceans, insects, and fungi and is considered the second most abundant polysaccharide. Commercially, most chitosan is obtained from food-processing wastes (crab, shrimp, or lobster), and the recent emergence of higher valued nutraceutical markets for both chitosan and its monomer glucosamine have stimulated worldwide production (*4*). The characteristic of chitosan that is most important for our study is that this polymer has nucleophilic primary amine groups at nearly every repeating sugar residue (since chitin deacetylation is incomplete, chitosan is formally a copolymer of glucosamine and *N*-acetylglucosamine). The reactivity of these amine groups is convenient as they allow chitosan derivatives to be generated with a range of facile chemistries (*5*) or, in our case, biochemistries.

The enzyme for our study is mushroom tyrosinase. Tyrosinases (and the related phenol oxidases) are ubiquitous oxidative enzymes that convert phenols into *o*-quinones that can undergo nonenzymatic reactions with a variety of nucleophiles. Impor-

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tantly, tyrosinases react with a broad range of phenolic substrates and phenols are abundant in nature (including in foods) (*6*). Reactions involving enzymatically generated quinones are responsible for a range of commonly observed phenomena including the enzymatic browning of food, insect sclerotization (*7*, *8*), and the setting of mussel glue (*9*−*11*). In previous studies, we showed that tyrosinase could react with a range of low molecular weight natural phenols and the “activated” quinones could be grafted onto chitosan. These grafting reactions lead to dramatic changes in the functional properties of the polymer (*12*−*15*). In addition to reacting with low molecular weight phenols, tyrosinase is also able to react with accessible tyrosine residues of peptides and proteins (*11*, *16*, *17*). In previous studies, we observed that tyrosinase could initiate reactions that lead to the grafting of gelatin chains onto chitosan (*18*) and that distinct functional properties were observed for these grafted copolymers (*19*, *20*).

Here, we extend our work in two important ways. First, we examine the grafting of peptides onto chitosan. Specifically, we grafted peptides obtained from acid-hydrolyzed casein. Casein is a milk protein and its hydrolysates have been exploited for a range of applications (*21*). Second, we show that tyrosinase-catalyzed grafting can be performed in the presence of a complex mixture of peptides and that prior purification is unnecessary. Tyrosinase chemoselectively reacts with peptide fragments containing accessible tyrosine residues and only these fragments are “activated” for grafting onto chitosan. Although it is impossible to completely control reactions of the “activated” peptides, we expect the quinones will preferentially react with chitosan due to the abundance and reactivity of their nucleophilic primary amines. Because of the lower pK_a values of chitosan’s primary amines (*22*, *23*), we expect them to be more reactive than either the peptides’ N-terminal amines or the ϵ-amines of lysine residues. The goals of this study are to demonstrate that peptides can be enzymatically grafted onto chitosan, and that the peptide-modified chitosans offer interesting viscoelastic properties.

MATERIALS AND METHODS

Chitosan from crab shells (85% deacetylation, molecular weight 300 000 as reported by the supplier), glucosamine, the dipeptide tyrosine-alanine (Tyr-Ala), and mushroom tyrosinase (2590 U/mg as reported by the supplier) were purchased from Sigma-Aldrich Chemicals. Acid-hydrolyzed casein was obtained from DMV International Nutritionals.

A concentrated chitosan “stock” solution was prepared by mixing chitosan flakes with water and intermittently adding small amounts of 2 M HCl to maintain a pH of 3. After the solution was mixed overnight, undissolved materials were removed by filtration, the solution was diluted to 1.6 w/v % chitosan, and the pH was adjusted to 6.0 with 1 M NaOH.

Reactions with small molecule analogues were performed by mixing the dipeptide Tyr-Ala (1 mM final concentration) with glucosamine (10 mM) and tyrosinase (60 U/mL). The reaction mixtures were incubated overnight after which the products were analyzed by electrospray mass spectrometry (ESMS). The ESMS spectra were obtained on a Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) as described previously (*13*). Reactions between the Tyr-Ala dipeptide (3 mM final concentration) and chitosan (0.32%, 20 mM repeating sugar unit) were performed with tyrosinase (60 U/mL) under homogeneous conditions (i.e. at pH 6 so all components were soluble). During the course of the reaction, samples were taken from the reaction mixture and the viscosity was measured at a constant shear rate (1.5 s^{-1}).

A variety of protein hydrolysates were initially examined. Acid-hydrolyzed casein was selected as a convenient system for detailed study because it was not precipitated by the addition of chitosan. Additionally, we report results for reactions with only a single concentration of casein hydrolysates (0.5 w/v %). Reactions with higher casein hydrolysate concentrations resulted in chitosan derivatives that could not be completely dissolved and our interest was to examine the behavior of polymer solutions and not polymer suspensions. For these reactions, we incubated chitosan (0.32 w/v %) with acid-hydrolyzed casein (0.5 w/v %) and tyrosinase (60 U/mL) overnight at room temperature with stirring. Following incubation, the casein hydrolysate-modified chitosan was precipitated from solution by adjusting the pH to about 8 with 1 M NaOH. The precipitate was collected by centrifugation, and the pellet was washed several times with distilled water. This partially purified material was dissolved in a 0.5 v/v % acetic acid solution to varying polymer concentrations. To determine the polymer concentrations in this solution, aliquots of known mass were dried in an oven (less than 60 °C) and the residue was weighed.

Various rheological tests were performed to examine the behavior of the peptide-modified chitosan. The tests were conducted on a ThermoHaake RheoStress1 rheometer, using a cone-and-plate geometry (60 mm diameter, 1° cone angle). The viscosity was measured as a function of the polymer concentration by using a constant shear rate of 1.5 s^{-1} for 2 min per sample. The viscosity was also measured as a function of shear rate between 1 and 100 s^{-1}. Finally, the elastic and viscous moduli were determined from a frequency sweep ($\omega = 0.1-10$ rad/s) at a controlled stress of 0.5 Pa. These measurements were conducted within the linear viscoelastic range of the sample probed, as verified by stress sweep measurements.

RESULTS

Evidence for Tyrosinase-Catalyzed Peptide Grafting. To obtain chemical evidence that tyrosinase can activate peptides for grafting, we performed reactions with small molecule models and analyzed the products using electron spray mass spectrometry (ESMS). Specifically, we incubated the dipeptide Tyr-Ala with the monomer glucosamine in the presence of tyrosinase. **Figure 1a** shows the magnified ESMS spectra for the glucosamine control. The prominent peaks at 179.9 and 162.1 are consistent with expectations for unreacted glucosamine (180) and a glucosamine that has lost a water molecule (162). Peaks showing loss of water from glucosamine residues have been reported previously (*13*, *24*). The peak at 340.9 is consistent with a glucosamine dimer that may be present as a contaminant. We do not have assignments for the peaks at 358.6 and 380.7 observed in **Figure 1a**; however, we have seen these peaks repeatedly for this batch of glucosamine.

Figure 1b shows the magnified ESMS spectra for the control prepared by incubating the Tyr-Ala dipeptide with tyrosinase. The peak at 253.3 is consistent with the unreacted Tyr-Ala dipeptide (252) while the peak at 504.9 is also observed in the spectra for the dipeptide alone (i.e. without tyrosinase). The peaks in **Figure 1b** that appear at m/z values of 526.9 and 800.6 may be quinone coupling products. Specifically, if the dipeptide is oxidized to convert the tyrosine to DOPA residues and these dipeptides are coupled, then we would expect coupling products to be spaced 270 m/z units apart.

Figure 1c shows the ESMS spectra for the sample in which glucosamine and the dipeptide were incubated with tyrosinase. The unique peak that appears in this spectrum is at 431.6. This m/z value is consistent with an adduct formed between the activated dipeptide and glucosamine. Such an adduct could be either Schiff base or a Michael-type adduct that has lost a water (*13*, *24*). **Scheme 1** shows a putative structure for the glucosamine-dipeptide adduct. This structure is analogous to other proposed product structures although we should note that the position of the linkage on the aromatic ring is uncertain (*24*) and the oxidation state of the adduct may vary. In conclusion,

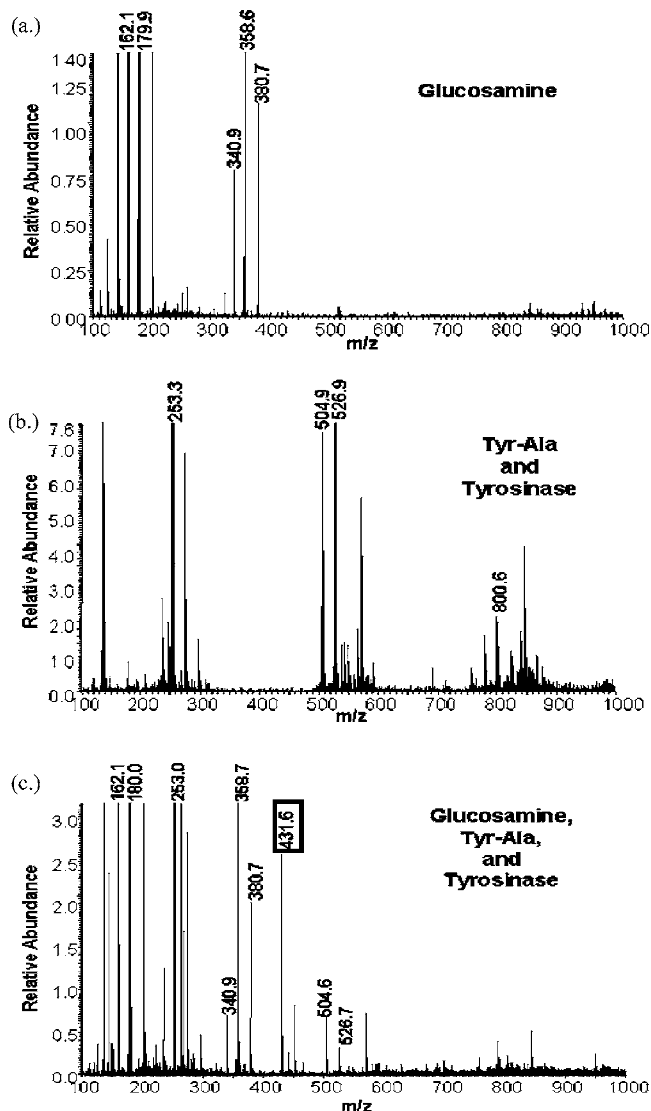


Figure 1. Chemical evidence for tyrosinase-initiated grafting by electrospray mass spectrometry: (a) control of glucosamine (10 mM); (b) control of Tyr-Ala (1 mM) incubated with tyrosinase (60 U/mL); and (c) product mixture obtained by incubating glucosamine (10 mM) and Tyr-Ala (1 mM) with tyrosinase (60 U/mL).

ESMS results indicate that tyrosinase can initiate conjugation of the model dipeptide with glucosamine.

Physical measurements were used to provide evidence that tyrosinase can initiate grafting of the Tyr-Ala dipeptide onto the chitosan polymer. For this, we incubated Tyr-Ala with chitosan and tyrosinase, and intermittently measured the viscosity. **Figure 2** shows that over the course of 4 h, the viscosity of the solution increased from 0.05 to 2.7 Pa•s. Visually, we observed that this solution changed from colorless to dark brown during the incubation. **Figure 2** shows no viscosity increase for the control solution containing the dipeptide and chitosan (but not tyrosinase). Additionally, the control solution was observed to remain colorless during the course of the experiment. Thus, the results in **Figures 1** and **2** indicate that tyrosinase can initiate the grafting of this model peptide onto chitosan.

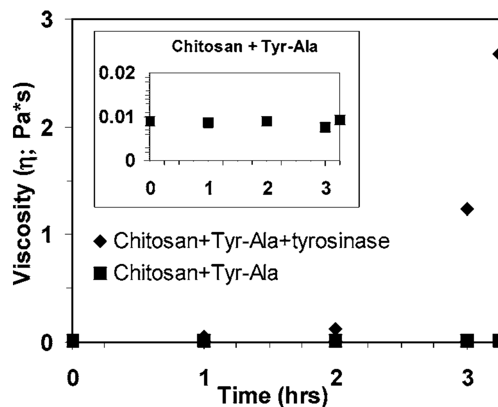
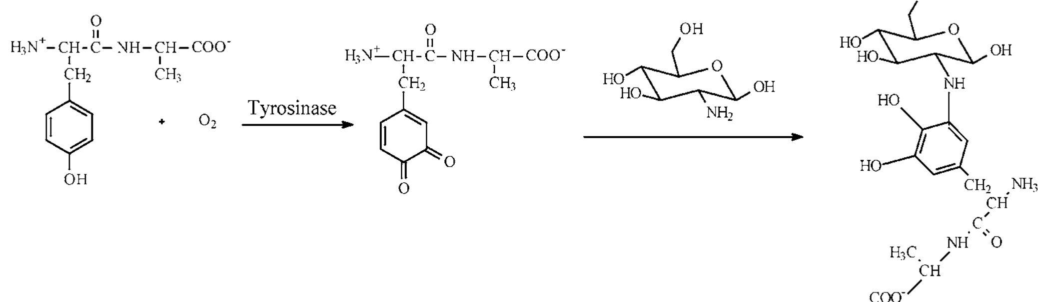


Figure 2. Physical evidence for tyrosinase-initiated grafting by rheometry. Homogeneous reactions were performed in solutions containing the dipeptide Tyr-Ala (3 mM), chitosan (0.32%, equivalent to 20 mM repeating units), and tyrosinase (60 U/mL) at room temperature and pH 6. The "control" solution was incubated without tyrosinase. The insert shows an expanded scale for the viscosity of the control.

Preparation of Peptide-Modified Chitosan from Casein Hydrolysate. In subsequent studies, we prepared peptide-modified chitosan by mixing acid-hydrolyzed casein (0.5 w/v %) with chitosan (0.32 w/v %) and tyrosinase, and incubating overnight. During the course of the reaction, the solution changed from colorless to dark brown, indicating oxidation of the casein hydrolysate. Additionally, we observed an increase in viscosity during the reaction analogous to that observed with the dipeptide (e.g. **Figure 2**). After reaction, the modified chitosan was precipitated by increasing the pH to 8, and the precipitate was extensively washed with water to remove ungrafted peptides. For measurement of their rheological properties, the peptide-modified chitosan was then dissolved in dilute acetic acid (0.5 v/v %). Three separate batches of peptide-modified chitosans were prepared and all measurements were performed with each batch. The rheological behavior was reproducible and representative results are presented below.

Rheological Properties of Peptide-Modified Chitosan. In initial studies, the polymer was dissolved at different concentrations and the viscosity was measured at a shear rate of 1.5 s^{-1}. **Figure 3** shows that the viscosity of the peptide-modified

Scheme 1. Proposed Tyrosinase-Initiated Reaction between Glucosamine and Tyr-Ala



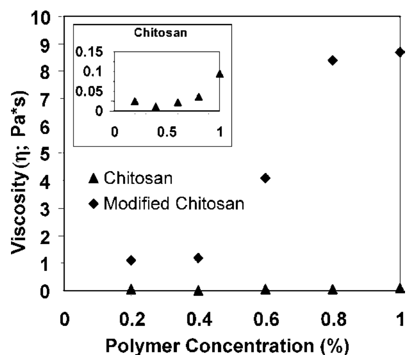


Figure 3. Viscosifying effect of the peptide-modified chitosan derivative. The modified chitosan was prepared by reacting acid-hydrolyzed casein (0.5 w/v %) with chitosan (0.32 w/v %) and tyrosinase (60 U/mL). After partial purification, the polymer was dissolved to different polymer concentrations in 0.5 v/v % acetic acid solution. The insert shows an expanded scale for the viscosity of the chitosan control.

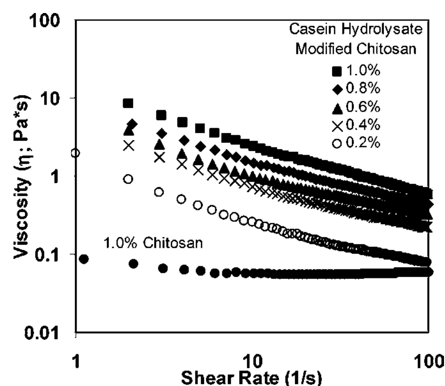


Figure 4. Shear thinning properties of solutions containing the peptide-modified chitosan derivative. The modified chitosan was prepared as described in the legend of **Figure 3** and dissolved to differing polymer concentrations. The viscosity was determined from rotational studies.

chitosan samples increased dramatically when the polymer concentration was increased above 0.2%. This increase in viscosity occurs at concentrations close to chitosan's overlap concentration (chitosan's c* is approximately 0.1% (*25*)). Compared to the peptide-modified chitosan, **Figure 3** shows that the viscosity for the unmodified chitosan control was considerably less sensitive to polymer concentration for the concentration range examined.

These samples were further analyzed by using rotational measurements over a range of shear rates ($1-100\ s^{-1}$). **Figure 4** shows that the peptide-modified chitosan samples had considerably higher viscosities compared to the unmodified chitosan control. Additionally, **Figure 4** shows that solutions of the peptide-modified chitosan were shear thinning even at a polymer concentration of 0.2%, while the 1% solution of the unmodified chitosan control displayed nearly Newtonian behavior.

To further examine the rheological behavior, we subjected samples to oscillatory shear. **Figure 5** shows that a peptide-modified chitosan sample at a concentration of 1% exhibits gellike elastic behavior. Specifically, the elastic modulus (G′) is greater than the viscous modulus (G″) and both moduli are nearly independent of frequency (ω) over the range of frequencies probed. This shows that the relaxation time of the sample is very high and possibly infinite, which suggests that a sample-spanning network of polymer chains is present in the sample. At lower polymer concentrations close to overlap (0.2%) the sample behaves like a viscous solution: in this case, G″ exceeds

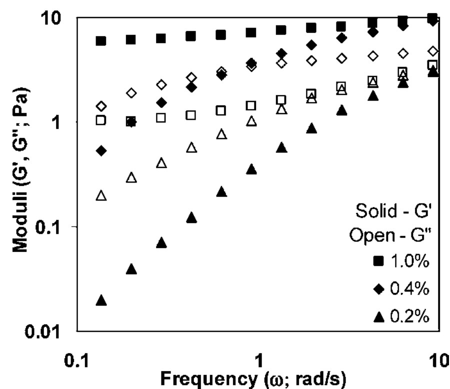


Figure 5. Viscoelastic behavior of the peptide-modified chitosan derivative. The modified chitosan was prepared as described in the legend of **Figure 3** and the moduli were determined from oscillatory measurements (ω = 0.1–10 rad/s) at a controlled stress of 0.5 Pa.

G′, and both moduli increase with ω. At an intermediate concentration (0.4%), the sample's behavior is viscoelastic: at high frequencies (short times) it responds elastically and at low frequencies (long times) it behaves as a viscous liquid. The relaxation time t_R of this sample is approximately 1 s, as discerned from the crossover frequency ω_c where G′ and G″ intersect ($t_R = 1/\omega_c$). In sum, the rheological measurements demonstrate that the peptide-modified chitosan confers viscoelastic properties to solution.

DISCUSSION

Here we report that tyrosinase can "activate" peptides for grafting onto the polysaccharide chitosan. This result is consistent with previous studies which demonstrated that tyrosinase can "activate" phenols for covalent grafting to glucosamine oligomers (*24*) and to chitosan (*12*, *13*, *26*). The chemoselectivity of the enzyme and the relative reactivity of chitosan's amines enable this grafting to be performed even in the presence of a complex hydrolysate mixture. Since peptide purification is not required, this grafting approach provides a simple means to exploit acid-hydrolyzed caseins for the chemical modification of chitosan.

The tyrosinase-initiated reactions are expected to add side groups or side chains onto the linear chitosan backbone although the size of the peptide branches and the degree of substitution will be difficult to characterize or control. Studies with well-defined synthetic polymers indicate that the addition of long branches onto a linear polymer backbone can confer viscoelasticity through enhanced entanglements (*27*−*29*). Additionally, polymers with short branches can confer viscoelasticity if the branches can undergo intermolecular associations that lead to physical cross-linking. A typical example of an associating polymer is a hydrophobically modified water-soluble polymer that has hydrophobic alkyl branches on the polymer backbone. These branches can associate through hydrophobic interactions with branches from other polymer chains to form transient network junctions (i.e. physical cross-links) that confer viscoelasticity even at relatively low polymer concentrations (*30*−*35*). In well-defined cases it may be possible to isolate the individual roles of entanglements or associations in conferring viscoelasticity (*36*−*41*). The macromolecular architecture and viscoelastic properties of branched biopolymers (e.g. mucins, proteoglycans, and gums) are seldom so well-understood (*42*, *43*). Nevertheless, biopolymers with branched architectures have also been observed to confer viscoelastic properties to aqueous solutions (*44*−*46*). In our studies, we observed that the peptide-

modified chitosans confer viscoelasticity to solution and these results are consistent with the generation of peptide branches that undergo associations to create physical network junctions.

Polymers that confer viscoelasticity to solutions are important for a variety of food, coating, and cosmetic products (*47*, *48*), and thus a range of methods has been developed to create such polymers. We believe there are three important aspects of the enzymatic method for creating viscoelastic chitosan derivatives. First, the three components (chitosan, protein hydrolysates, and tyrosinase) are all obtained from renewable resources and in some cases offer value-added byproduct opportunities for food-processing wastes. Additionally, we observed that the casein hydrolysate-modified chitosan derivative conferred viscoelastic properties even at low polymer concentrations. Although we expect protein hydrolysates from a variety of sources could be enzymatically grafted onto chitosan, we should recognize a few technical limitations. Hydrolysates with low tyrosine contents are expected to have low grafting efficiencies. Hydrolysates with large and/or acidic peptides may form insoluble precipitates with chitosan. Finally, some hydrolysates may contain peptides that lead to the cross-linking (and not branching) of chitosan.

The second important aspect of this work is that the peptide-modified chitosan is prepared under simple and safe conditions. Specifically, tyrosinase is used for the in situ activation of the peptides for grafting onto chitosan's amine groups. This can be contrasted with the chemical modification of polysaccharides that lack amine groups (e.g. cellulose). In the absence of amines, reactive reagents (e.g. acid chlorides or epoxides) are generally required to graft at the polysaccharide's hydroxyl groups. Since reactive reagents are not required for tyrosinase-catalyzed modification of chitosan, the safety concerns are considerably lessened, and simpler manufacturing facilities could be envisioned.

Finally, the peptide-modified chitosan derivatives obtained by tyrosinase should be environmentally friendly and biodegradable (*13*, *14*). In fact, these products may even be safe to consume given that all of the components (i.e. chitosan, casein hydrolysates, and mushroom tyrosinase) are derived from food manufacturing. Thus, we believe tyrosinase-catalyzed grafting of peptides onto chitosan may provide a "green" alternative for conferring viscoelasticity to aqueous solutions.

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