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# Laccase-mediated functionalization of chitosan with 4-hexyloxyphenol enhances antioxidant and hydrophobic properties of copolymer

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ABSTRACT

An effective method to functionalize chitosan with 4-hexyloxyphenol (HP) under homogeneous reaction conditions was developed using laccase as the catalyst. The resulting copolymer was characterized for chemical structure, grafted-HP content, surface morphology, thermal stability, antioxidant capacity, hydrophobic properties and tensile strength. Solid-state $^{13}C$ NMR spectrum confirmed the incorporation of HP onto chitosan. X-ray diffraction (XRD) showed a decrease in the degree of crystallinity for laccase/HP treated chitosan compared to pure chitosan. The grafted-HP content in laccase/HP-treated chitosan first increased and then declined with increase of the initial HP/chitosan ratio. A heterogeneous surface with spherical particles on the laccase/HP treated chitosan was observed by environmental scanning electron microscopy (ESEM) and scanning probe microscopy (SPM). The laccase/HP treatment of chitosan improved the thermal stability of copolymer. More significantly, the HP functionalized chitosan showed greatly improved $ABTS^{\cdot+}$ and DPPH radicals scavenging capacity, compared with pure chitosan. The hydrophobicity property of the HP functionalized chitosan also significantly increased although its tensile strength decreased. This new type of composite with double functionalities (i.e., antioxidant and hydrophobic) could potentially be used as food packaging materials or coating agents.

## 1. Introduction

As a natural resource, chitosan derived from the deacetylation of the chitin has several unique properties such as biocompatibility, antimicrobial, film-forming capacity, and low toxicity. Chitosan and its derivatives have been widely studied in a variety of applications such as food packing or preservation (Li et al., 2011; Wu et al., 2016), metal ions adsorption (Ayoub et al., 2013), adhesives (Mati-Baouche et al., 2014; Patel, 2015), dye removal, and pharmaceutical applications (Zou et al., 2016). Particularly due to its good film-forming capacity, chitosan can be used to fulfill the needs of various packaging requirements in the form of transparent films or coatings to improve the quality of fresh food and extend food shelf life (Arancibia et al., 2014). The antioxidant capacity of native chitosan may be due to the capacity of residual free amino groups of chitosan to react with free radicals forming stable macromolecular radicals and ammonium groups (Yen et al., 2008). However, chitosan lacks a hydrogen atom that can be easily donated in order to serve as a good antioxidant (Schreiber et al., 2013). The low antioxidant activity of chitosan is a major problem affecting food quality and biological applications (Nunes et al., 2013). The application of chitosan has also been restricted due to its hygroscopicity, which has been attributed to the reactive amine groups especially in humid environments or in an acid media (Dutta et al., 2002). Phenolic compounds, classified as primary antioxidants, readily scavenge free radicals by donating a hydrogen atom or an electron (Schreiber et al., 2013). Therefore, introducing a hydrophobic phenolic compound onto chitosan would provide a new chitosan derivate with antioxidative and hydrophobic properties.

Chemical functionalization of chitosan can improve the antioxidant capacity (Hu et al., 2016; Schreiber et al., 2013; Xie et al., 2014) and hydrophobic property (Höhne et al., 2007; Pradal et al., 2011) of chitosan composites by the incorporation of small functional groups onto chitosan. However, chemical functionalization typically has several drawbacks, including the requirement of reactive reagents and harsh experiment conditions, as well as the use of crosslinking agents may lead to toxic side effects (Lin et al., 2005). Chemo-enzymatic modifications provide an alternative method for functionalization of chitosan that could avoid the safety and environmental concerns mentioned above (Aljawish et al., 2015). Research efforts have been conducted regarding the improvement of the antioxidant activity of

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chitosan-based polymers through enzymatic grafting of phenolic compounds (Aljawish et al., 2016; Hu and Luo, 2016). This type of enzymatic modification of chitosan is usually carried out using polyphenol oxidases such as tyrosinase, peroxidase, and laccase (Aljawish et al., 2015). Tyrosinase has been used to graft flavonoids or 4-hexyloxyphenol (HP) onto chitosan, conferring new capacities to chitosan such as antioxidative, antibacterial (Sousa et al., 2009) and hydrophobic properties (Chen et al., 2000). Tyrosinase-mediated chitosan derivatives can also be used as functional coatings for food packaging materials (Liu et al., 2014). Peroxidase has also been used to graft phenolic compounds onto chitosan to enhance its antioxidative (Zavaleta-Avejar et al., 2014), antibacterial (Sakai et al., 2014), and hydrophobic properties (Vachoud et al., 2001). Laccases are blue multi-copper oxidases and have shown encouraging potential as biocatalysts in organic synthesis mainly because the catalytic reaction only requires oxygen molecular and releases water as the only by-product (Kudanga et al., 2017). The laccase activated phenolics are potential cross-linkers of chitosan as a novel approach to synthesizing chitosan hydrogels (Huber et al., 2017). There have been several investigations on laccase-catalyzed functionalization of chitosan with phenolic compounds to enhance the antioxidative activity (Aljawish et al., 2012; Božič et al., 2013), antibacterial activity (Aljawish et al., 2014b; Božič et al., 2012), and barrier properties (Aljawish et al., 2016).

In previous studies of chitosan-phenolic-enzyme systems, the hydrophobic functionalization of chitosan was achieved using peroxidase (Vachoud et al., 2001) or tyrosinase (Chen et al., 2000; Liu et al., 2014) as the catalyst. In terms of functionalization of chitosan with phenolic compound by laccase, most of the studies focused on improving the antioxidant properties of chitosan derivate. Furthermore, most of the phenolic compounds being used in these studies contained carboxyl group or at least two phenolic hydroxyl groups, and were soluble in water. However, to the best of our knowledge, few publications have reported on the functionalization of chitosan with hydrophobic monophenols by laccase targeted at improving the antioxidant and hydrophobic properties of chitosan at the same time.

The objective of this work is to functionalize chitosan with HP under homogeneous conditions for enhancing the antioxidant and hydrophobic properties of chitosan through the laccase-mediated coupling reactions. The incorporation of HP into chitosan was supported by solid-state $^{13}C$ nuclear magnetic resonance ($^{13}C$ NMR) spectroscopy studies. The surface morphology of the chitosan and its derivative were further characterized by environmental scanning electron microscope (ESEM) and scanning probe microscope (SPM). The crystallographic structure and the thermal behavior of chitosan derivatives were analyzed by X-ray diffraction (XRD) and thermogravimetric analysis (TGA), respectively. Finally, the chitosan and its derivative films were characterized in terms of antioxidant, hydrophobic, and mechanical properties.

## 2. Materials and methods

### 2.1. Chemicals

Chitosan with a degree of de-acetylation of 85% was obtained from Sinopharm Chemical Reagent Co. Ltd. 4-Hexyloxyphenol (HP), 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were all purchased from Sigma-Aldrich. All other reagents were of the analytical grade and used as received.

### 2.2. Laccase

Laccase (NS51003) from *Ascomycete myceliophthora thermophila* was provided by Novozymes (Tianjin, China) with a specific activity of 1000 U/ml. 1 U was defined as the amount of enzyme that oxidizes 1 μmol of ABTS per minute in a sodium acetate buffer (pH 4.5) at 25 °C.

### 2.3. Enzymatic functionalization of chitosan

Enzymatic functionalization of chitosan was performed according to a modified homogeneous grafting method (Božič et al., 2012). First, the chitosan solution (0.3% w/v, dissolved in 0.1 M acetate buffer solution, pH 4.5) and the HP solution (10% w/v, dissolved in methanol) were prepared accordingly. The chitosan solution was heated to 45 °C. The HP solution was then added into the chitosan solution under a constant stirring rate. The ratio of the HP to chitosan was 1:2 (w/w). Afterwards, laccase (200 U/g HP) was slowly dropped into the chitosan-HP mixture solution. The laccase dosage used in this paper was optimal for improving the antioxidant properties of chitosan derivates. The laccase/chitosan-HP mixture was allowed to react for 15 h at 45 °C. The resultant solution was centrifuged at 5000 rpm for 5 min to remove the solid residue. 1 M NaOH solution was added to the supernatant to precipitate the solid product. After centrifugation, the precipitation was extracted with methanol for 12 h to remove any unreacted HP and then washed extensively with distilled water to remove the methanol. The resulting samples were lyophilized and stored at room temperature. The pure chitosan and the HP-chitosan solutions were prepared following the same procedures except the steps involving laccase were skipped.

### 2.4. Preparation of chitosan films

The chitosan solution was prepared by dissolving 0.10 g chitosan particles in 10.00 ml of acetic acid solution (2%, v/v) according to a method described by Aljawish et al. (Aljawish et al., 2014c). Chitosan films were formed by adding 5.00 ml of the above solution to a 7 cm-diameter Petri dish, and the solution was then oven-dried overnight at 45 °C. The dried films were neutralized by immersion in 1 M NaOH solution for 3 h, thoroughly washed with methanol and water, and then dried overnight at 40 °C. The untreated chitosan and HP-treated chitosan were used as controls. All the film samples were placed in sealed polyethylene bags and stored at room temperature.

### 2.5. Chemical evidence for chitosan functionalization

The structural characteristics of chitosan and its derivatives were analyzed by solid-state $^{13}C$ NMR to confirm the grafting of HP onto chitosan by laccase. Solid-state $^{13}C$ NMR experiments were performed on an AVIII 400 MHz WB spectrometer (Bruker Inc., Germany) operating at a $^{13}C$ frequency of 100.62 MHz equipped with a double resonance H/X CP-MAS 4 mm probe. The crystallinity of chitosan was recorded by a Brooke D8-ADVANCE X-ray diffractometer (AXS Company, Germany) over a 2θ range from 3° to 50°.

### 2.6. Determination of 4-hexyloxyphenol content onto chitosan derivative

The content of HP in chitosan composite was indirectly determined by measuring the content of the remaining HP in the reaction medium according to a modified Folin-Ciocalteu assay method (Liu et al., 2013b). Briefly, ~10 mg of chitosan derivative was dissolved in 50 ml of aqueous acetic acid (0.5%, v/v). ~1 ml of sample solution was mixed with 1 ml of Folin-Ciocalteu reagent (10 times dilution) and allowed to react at 30 °C for 5 min in the dark. Then, ~5 ml of saturated $Na_2CO_3$ solution was added and mildly shaken periodically for 1 h before measuring the UV absorbance at 760 nm. HP was used to calculate the standard calibrations curves. The content of HP in grafted chitosan was expressed as mg of HP per g of laccase/HP-chitosan (mg HP/g). The grafting percentage of HP was then calculated from the HP content in the laccase/HP-chitosan and the utilized amount of HP in the preparation of laccase/HP-chitosan. Each point represents the average of three independent experiments.