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Incorporation of lysozyme-rectorite composites into chitosan films for antibacterial properties enhancement

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ABSTRACT

The demand for designing antibacterial materials was quite substantial in packing and biomedical materials fields. Chitosan had a wide utilization to satisfy this demand. In this study, by incorporating lysozyme (LY) – rectorite (REC) into chitosan films, the ultimately obtained hybrid films can own the enhanced antibacterial properties and still remains good mechanical properties. Scanning electron microscopy (SEM) images revealed that LY and REC could be homogeneously distributed in the CS films. X-ray photoelectron spectroscopy (XPS) and Energy-dispersive X-ray analysis (EDX) results verified that the LY-REC incorporation process was successful. Small angle X-ray diffraction (SAXRD) and Fourier transform infrared (FT-IR) spectra revealed that some intercalation reactions occurred between CS chains and REC. The hydrophobic properties of the CS films were increased by the addition of LY and REC, determined by water contact angle measurement. In comparison with CS films, the mechanical properties of the composite films after adding LY-REC were reduced by 27.58%, but still maintained high tensile strength. Besides, the antibacterial properties of the films could be enhanced by introducing LY-REC. This method exhibited great application value in the food packaging fields.

1. Introduction

Food contamination is mainly caused by undesirable microorganisms [1]. To inhibit such contamination, packaging films with good antimicrobial properties have been developed [2]. Particularly, the development of degradable biomass derived from natural origins is more and more popular in packaging films fabrication because it is easy to be obtained and low cost [3]. Therefore, there is a great potential to search biodegradable, non-toxic, antimicrobial and edible materials for food packaging [4].

Antimicrobial food packaging is studied by combining the antimicrobial materials into polymer films to inhibit the growth of microorganism [5]. Lysozyme (LY), as a kind of natural and antibacterial protein, is extensively existence in egg white proteins, human tears and saliva, et al. [6]. LY has good antibacterial property because it can hydrolyze the insoluble polysaccharides of bacterial cell walls, especially the Gram-positive bacteria [7,8]. LY as non-toxic protein, can be easily digested and absorbed by the human body without any side effects [9]. It was reported that the antimicrobial films prepared by incorporating LY into cellulose acetate not only effectively maintain the LY activity, but also improve the antimicrobial ability of LY [10]. Besides, it has been reported that the antibiotic property of LY immobilized onto chitin nanowhiskers was greater than that of free LY [11]. However, due to its poor film-forming ability, the application in food packaging fields has been limited. Therefore, it is a novel strategy to incorporate LY into a packaging film to expand the application scope of LY.

Chitosan (CS) is a natural biological macromolecule derived from chitin by chemical deacetylation, which is the second most abundant natural polymer after cellulose [12]. CS is the only alkaline polysaccharide in the nature. Owing to the good biocompatibility, low toxicity, biodegradability and film-forming property, CS has gained great attentions in food packaging field [13,14]. The antibacterial ability of CS is related to the protonation of amino group. Besides, the positive amino groups of CS could interact with

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the negatively charged surface of the microorganisms cells, leading to their death [15]. For this reason, CS has been successfully applied to prolong the storage life of fresh cut broccoli [16], longan fruit [17] and many other vegetables [18].

Recent studies reported that polymer/layered silicate composites had better performance [19,20]. Rectorite (REC), a kind of layered silicate, possesses a wide application prospect for the sandwich structure, including adsorption [21], bacteria inhibition [22], drug controlled release [23] and tissue regeneration [24]. REC has no antimicrobial activity, but it has the adsorption and stabilizing effect on bacteria when it combines with other antibacterial materials [25]. Previous articles have reported that CS/REC composites improved the antimicrobial effect of CS by adsorbing the bacteria on the surface of REC, and then exposed to the matrix of CS [26,27]. Due to the good chemical stability and heat-resisting function, REC can improve the strength and thermal property of composites. Therefore, REC was also been elected as a candidate to enhance the application performance of composite films.

The objective of this study was to develop CS/LY/REC films with high strength and enhanced antibacterial properties via a green method. Briefly LY and REC were added into the CS solutions, and then the films were fabricated by tape casting technique. The morphology, structure, antibacterial activities, thermal and mechanical properties of the prepared films were investigated. Such simple and green method could be used to fabricate composite materials with strong antibacterial properties, which could broaden the applications in the fields of food packaging and biomedical materials.

2. Materials and methods

2.1. Materials

CS (M_w = 200 KDa, DD = 92%) was provided by Yuhuan Ocean Biochemical Co., China. Calcium rectorite (Ca^{2+}-REC) was supplied by Hubei Mingliu Co., China. LY (activity 25,000 U mg^{-1}) was obtained from Amresco Co., USA. Acetic acid was purchased from Aladdin Chemical Reagent Co., China. All aqueous solution were prepared by purified water with a resistance of 18.2 MΩ cm. *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) were obtained from China Center for Type Culture Collection at Wuhan University (Wuhan, China).

2.2. Preparation of various films

CS (600 mg) was dissolved in 1% aqueous acetic acid to obtain 3% (wt%) CS solution. LY (600 mg), REC (50 mg) or LY/REC (600 mg LY and 50 mg REC) composites were added slowly into the as-prepared CS solution, respectively. All the resulted mixtures were vigorously stirred for 24 h at room temperature. The achieved mixtures were poured on the surface of glass plates (10 × 10 cm^2) to form the films. When the films were semi-dry, they were immersed in 1 M NaOH for 30 min. Thereafter, all films were washed with purified water three times to remove the excess NaOH. Finally, the films were dried in oven at 45 °C for further characterizations.

2.3. Characterization

The morphology and composition of as-prepared films were analyzed using field emission scanning electron microscopy (FE-SEM) and energy-dispersive X-ray (EDX) spectroscopy (S-4800, Hitachi, Japan). The X-ray diffraction (XRD) was carried out using a diffractometer type D/max-rA (Rigaku Co., Japan) with Cu target and Kα radiation (λ = 0.154 nm). X-ray photoelectron spectroscopy (XPS) was used to identify elemental compositions on the surface of films by using an axis ultra DLD apparatus (Kratos, UK). The Fourier transform infrared (FT-IR) spectra were recorded with a Nicolet 170-SX (Thermo Nicolet Ltd, USA). Moreover, the films were measured for a stress-strain response using a tensile tester (ETM502A, Shenzhen wance Instrument Co., Ltd., China).

2.4. Measurement of LY activity

The activity of free LY was measured by the spectrophotometric turbidity method described as the previous literature [28]. The samples were prepared by immersing the chitosan hybrid films in 20 mL of 0.15 *M* phosphate buffer (pH 6.2) in vials and shaken at room temperature. A substrate solution of *Micorcoccus lysodeikticus* cells was prepared in 0.15 *M* phosphate buffer solution. 3 mL of *M. lysodeikticus* solution was added in a cuvette, mixed with 20 μL of the samples solution, and then immediately examined the absorbance at 450 nm for 30 s by a visible spectrophotometer (722, Tianjin Precise Instrument Co., Ltd., China). Activity of lysozyme was monitored by measuring the decrease in solution absorbance at 450 nm.

$$\text{Activity}\left(\text{Umg}^{-1}\right) = \frac{\Delta \text{OD}_{450}}{0.001\text{m}} \quad (1)$$

where ΔOD_{450} referred to the reduction of absorbance at 450 nm of *M. lysodeikticus* solution/min, m was the mass (mg) of LY in 20 μL of the samples solution. One activity was expressed in terms of units of activity per milligram. All samples were texted in triplicate.

2.5. Microbial inhibition assay

The shake flask method was performed to investigate the antibacterial activity of the films [29,30]. Gram-negative bacterium *E.coli* and Gram–positive bacterium *S.aureus* were chosen as the representative microorganisms and cultivated in culture medium in an incubator. The films were randomly cut to disks (diameter = 6 mm) and sterilized under ultraviolet radiation lap for 30 min. For the antibacterial assay, randomized three disks were immersed in 2 mL of diluted bacteria medium, and then incubated at 37 °C for 18 h with agitation. Another 2 mL of diluted bacteria medium was also incubated under the same conditions as control group. The concentration in the microorganism suspension was 10^6 CFU/mL. The count of bacterial colonies were determined via a plate count method [31]. We could evaluate the bacterial inhibition activity from the following equation:

$$\text{Antimicrobial activity}\,(\%) = \frac{A - B}{A} \times 100\% \quad (2)$$

where A and B were the number of surviving microorganisms in the control and test samples, respectively.

3. Results and discussion

3.1. Morphology of CS, CS/LY and CS/LY/REC films

The morphology of various films casting with different solutions are shown in Fig. 1. Obviously, a smooth and continuous structure could be observed on the surface of CS films (Fig. 1a), which might be due to the good film-forming properties of CS. However, due to the effect of degradation of CS by LY, some tiny particles existed on the surface of the CS/LY films (Fig. 1b), resulted a little roughness appeared on the CS/LY films surface. In addition, after REC was added, the surface of CS/LY/REC films exhibited obviously irregular distribution and good stereoscopic effects, were full of small holes (Fig. 1c). The insoluble of REC seriously hampered the diffusion of solution on the surface of glass plates. The special structure of REC also had greatly affected the process of film-forming by hindering the free dispersal of CS chains.