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Synthesis, anti-oxidant activity, and biodegradability of a novel recombinant polysaccharide derived from chitosan and lactose

Ming Guo[a,c,*], Yanfei Ma[a,b,c], Chunge Wang[a,c], Hongzhi Liu[b,c,d,**], Qian Li[b,c], Meng Fei[a,c]

[a] Department of Chemistry, Zhejiang Agricultural & Forestry University, Hangzhou, Zhejiang, 311300, China
[b] School of Engineering, Zhejiang Agricultural & Forestry University, Hangzhou, Zhejiang, 311300, China
[c] National Engineering and Technology Research Center of Wood-based Resources Comprehensive Utilization, Zhejiang Agricultural & Forestry University, Hangzhou, Zhejiang, 311300, China
[d] Key Laboratory of Wood Science and Technology, Hangzhou, Zhejiang Province, 311300, China

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ABSTRACT

A novel recombinant polysaccharide (RP) based on polysaccharide–disaccharide was synthesized from oligo-chitosan (oligo-CS) and reducing lactose using Maillard reaction with the yield of 85.1%. Chemical structure and thermal stability of RP was characterized by Fourier transform infrared spectrum (FT-IR), solid-state nuclear magnetic resonance spectroscopy (CP/MAS ^{13}C-NMR), and thermo gravimetric analysis (TGA). The anti-oxidant activity of RP was preliminarily investigated by its scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Biodegradability of RP was also examined by the observation of growth status of *Aspergillus niger* colony. It was demonstrated that RP achieved excellent radical-scavenging efficiency (>80%) at high concentrations of DPPH and its scavenging ability was superior to that of CS, suggesting that anti-oxidant property of CS was remarkably promoted by chemical modification with reducing lactose via Maillard reaction. And biodegradation test revealed that RP had better biodegradability than CS.

1. Introduction

As one of the most promising materials, natural polymers have recently received increasing attentions due to their renewable, non-toxic, eco-friendly, and biodegradable benefits (Kim, Yun, & Ounaies, 2006; Malinconico, Cerruti, Santagata, & Immirzi, 2014; Thakur, Thakur, & Gupta, 2013). However, the unsatisfactory performance of naturally available polymers usually fails to meet the needs in different fields. In order to expand their range of applications, structure modification through semi-synthesis, whole synthesis, and combinatorial chemistry, is considered to be the effective ways in improving the performance of natural polymers (Beloshenko, Askadskii, & Varyukhin, 1998). As for these synthesis methods, small molecules tend to be grafted onto natural polymer chains. However, few restructuring studies of different natural polymers were reported due to the non-isotropic nature between their external structures.

Gene recombination refers to a process of two or more than two parents DNA that are combined to form new DNA molecular sequences (Jocelyn, Elliott, & Stephen, 2010), and is considered as one of the most important findings in the biological science. Its principle lies in the fact that extracted DNA fragments are covalently linked onto targeted DNA ones to obtain recombinant genes through the complementary pairing of base groups, independent distribution of genes, or exchange of linkage genes. Like DNA molecules consisting of different base units, polysaccharide is composed of monosaccharide unit. The similarity in the constructing rationale between polysaccharides and DNA has motivated us to consider whether the concept of gene recombination can be applied to the synthesis of recombinant polysaccharides (Wrodnigg & Stütz, 1999), which would be interesting from the viewpoint of designing new natural polymers.

Natural polysaccharides, such as chitosan (CS) and cellulose, are a class of very important polymers that have been widely utilized in a variety of fields. Chemical modification with functional molecules can impart improved performance to polysaccharides. Among various modification reactions, Maillard reaction is a chemical reaction

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[*] Corresponding author at: Department of Chemistry, Zhejiang Agricultural & Forestry University, Hangzhou, Zhejiang, 311300, China. Tel.: +86 571 63740852; fax: +86 571 63730919.
[**] Corresponding author at: National Engineering and Technology Research Center of Wood-based Resources Comprehensive Utilization, Zhejiang Agricultural & Forestry University, Hangzhou, Zhejiang, 311300, China. Tel.: +86 571 63740852; fax: +86 571 63730919.
E-mail addresses: tdky@hotmail.com (M. Guo), hzliu@iccas.ac.cn (H. Liu).

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between nucleophilic amino groups ($-NH_2$) and reactive carbonyl ones ($-C{=}O$) of reducing saccharides (Jing, Yap, Wong, & Kitts, 2009; Maillard, 1912; Rao, Chawla, Chander, & Sharma, 2011). CS contains the $-NH_2$ functional group, whereas semi-acetal groups of the saccharides can be converted to $-C{=}O$ under the specific condition. Thus, Maillard reaction between CS and reducing saccharides can be utilized to synthesize recombinant polysaccharides (RP). Since Maillard reaction products (MPRs) exhibited some extent of oxidant resistance, the study of their anti-oxidation activity has been attracting considerable interests (Azlan & Wan, 2009; Cai et al., 2009; Harish Prashanth & Tharanathan, 2007; Ma, Chen, & Zhao, 2013; Zhang, Yesim, Parveen, Dachao, & Stuart, 2010). It has been demonstrated that the anti-oxidation properties of chitosan after the modification of Maillard reaction were remarkably improved as compared with chitosan or N-alkylation chitosan derivative. To date, the majority of studies focused on Maillard reaction of chitosan with such mono-saccharides as glucose (Rao et al., 2011; Weerakkody, Labbett, Cheng, & Kosaraju, 2011) and fructose (Dong et al., 2014; Ying, Xiong, Wang, Sun, & Liu, 2011). Because of different chemical structure between mono-saccharides and disaccharides, it was expected that the MRPs from disaccharide and chitosan would display varying antioxidant activity from those from mono-saccharide and chitosan. However, the use of CS and reducing disaccharide containing reactive carbonyl groups (e.g. lactose) is scarcely reported in the literature so far.

In this work, a novel recombinant polysaccharide (RP) was successfully synthesized from lactose and the oligo-polysaccharide from chemical degradation of CS in terms of Millard reaction, and its chemical structure and thermal stability were characterized by means of FT-IR and CP/MAS ^{13}C-NMR, and TGA. And anti-oxidation and biodegradability of the resultant RP were evaluated as well.

2. Experimental

2.1. Materials

Chitosan (degree of deacetylation ≥90.0%, $M_w = 1.43 \times 10^5$ Da) was purchased from Sinopharm Chemical Reagent Co., Ltd. Lactose (purity ≥ 97.0%) was provided by Hongrun technology Co., Ltd. Hydrogen peroxide solution with H_2O_2 content of ≥30.0% was supplied by Xilong chemical Co., Ltd. All other reagents were analytical grade and used without further purification.

2.2. Synthesis of oligo-chitosan (oligo-CS) and recombinant polysaccharide (RP)

Chitosan (CS) solution was prepared by dissolving chitosan (2.0 g) in 150 mL acetic acid solution(1.0 wt%)under vigorous stirring. And 80 mL of 5 wt% H_2O_2 was slowly added to the CS solution and then refluxed at 60 °C for 4 h. After cooling to room temperature, the pH value of the mixture was adjusted to 10 using 10 wt% NaOH solution, and then concentrated under reduced pressure. Afterward, 2–3 times volume of anhydrous ethanol was added into the concentrate to allow the precipitation of the products overnight. The precipitate, was finally filtered off and dried at 50 °C under vacuum to obtain oligo-CS.

Oligo-CS (2.3 g) was dissolved in 50 wt% ethanol and lactose (1.0 g) was separately dissolved in 1 wt% acetic acid. A certain amount of lactose–acetic acid solution was added dropwise to oligo-CS–ethanol solution under stirring at 85 °C and then reflux for 8 h. After the completion of reaction, an excessive amount of anhydrous ethanol was added into the above mixture under stirring. After standing for 8 h, RP was recovered as the precipitate using the centrifugation (4000 rpm and 15 min), then adequately washed using anhydrous ethanol, and finally dried until the constant weight (yield: 85.1%).

2.3. Characterization

Molecular weight and its distribution index (PD = M_w/M_n) of the sample were determined by PL GPC 50 plus Gel Permeation Chromatography equipped with differential refractive detectors and PL Guard Column (10 μm) with H_2O as an eluent and poly(ethylene oxide) as standard substances. Column temperature and flow rate were set at 30 °C and 0.80 mL min^{-1}, respectively. Infrared spectra of oligo-CS, lactose and RP were recorded using Prestige-21 Fourier transform infrared spectrometer (FT-IR) in the wave number range of 500–4000 cm^{-1}. CP/MAS ^{13}C NMR spectra of CS and RP were recorded with AVANCE II/400 MHz nuclear magnetic resonance spectrometer. Fracture surfaces of the sample films were coated with gold and then examined on a SS-550 scanning electron microscope (SEM, Shimadzu, Japan) with an accelerating voltage of 10 kV. Thermal stability of samples was studied on a Netzsch STA 200 PC thermal analyzer (NETZSCH, Germany) under nitrogen atmosphere with a flow rate of 25 mL min^{-1}. A certain amount of samples (9.2–10.4 mg) were heated from 25 to 600 °C at a heating rate of 20 °C min^{-1}.

Radical-scavenging activity of the products was measured in the calorimetric cylinder equipped with 5 mL anhydrous ethanol solution of 1×10^{-4} mol L^{-1} 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Hatano et al., 1989). A certain amount of the samples (0.5–2.5 g) were added into the cuvette filled with the above solution. After left to stand for 30 min at room temperature in a dark room, the absorbance (A_i) at 517 nm was measured using UV-Vis spectrometer (UV-2450, Shimadzu, Japan). The absorbance of deionized water was measured as A_0, while the absorbance is measured as A_j when DPPH was replaced by anhydrous ethanol.

$$\text{DPPH} \quad \text{clearance} = (1 - (A_i - A_j)/A_o) \times 100\% \qquad (1)$$

For the biodegradation test, the CS and RP samples were examined as the sole carbon source medium with *Aspergillus niger* as a degrading strain material. The composition of *Agar* culture-medium is 15 wt% sample powder, 0.05 wt% $MgSO_4{\cdot}7H_2O$, 0.1 wt% NH_4Cl, 0.0005 wt% $CaCl_2{\cdot}2H_2O$, 0.554 wt% KH_2PO_4, and 1.194 wt% $Na_2HPO_4{\cdot}12H_2O$. After pH value of the culture medium was adjusted to 6.8-7.0 using 0.01 mol L^{-1} NaOH, a certain amount of sample powder was evenly spread on the surface of the culture medium. The strain was inoculated using the sterile gun in the center of the culture medium, which was then put into the oven at 28 °C for the cultivation. Growth status of colony was observed at some intervals to evaluate the biodegradability of samples.

3. Results and discussions

3.1. Synthesis of recombinant polysaccharide

Due to the presence of strong hydrogen bonding networks, chitosan (CS) is difficult to dissolve in common solvents except diluted acid, thus restricting the broad use of CS in many applications. Usually, physical degradation, chemical degradation, and bio-degradation were often employed to improve the solubility of CS (Cai et al., 2011; Gryczka et al., 2009; Patwardhana, Satrio, Brown, & Shanks, 2009; Wasikiewicz & Yeates, 2013; Zawadzki & Kaczmarek, 2010). Among them, chemical degradation is considered to be an ideal method for the preparation of oligo-CS (Sun, Zhou, Xie, & Mao, 2007) because of simple and convenient advantages. It was found that the oligo-meglumine sugar synthesized from the hydrolysis of CS showed unique functional properties and physiological activities. Molecular weight of oligo-CS was much decreased with respect to that of CS, and so the intermolecular

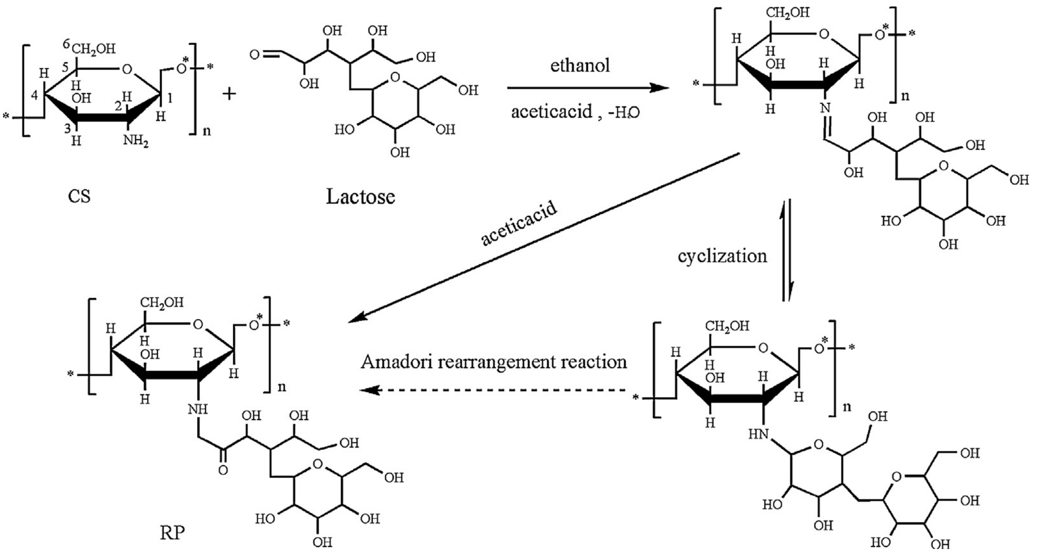

Fig. 1. Synthesis route of recombinant polysaccharide from oligo-CS and lactose via Millard reaction.

hydrogen bonding became weaker. Such changes in chain length and molecular conformation would increase its disorder degree in the aqueous solution. It is expected that more active groups (—NH_2) in the molecules would be involved in the Millard reaction with carbonyl groups (—C=O) of reduced lactose. In this study, RP was prepared from oligo-CS as an amino donor and lactose as a carbonyl acceptor through Maillard reaction and its synthetic route is schematized in Fig. 1.

3.2. Structure characterization of the RP

To determine the structure of the synthesized RP, its solid-state ^{13}C-NMR and FT-IR spectra were measured. Fig. 2 shows solid-state ^{13}C-NMR spectra of CS, lactose, and RP. In Fig. 2a, the peaks at 103.7, 84.1, 74.8, 60.1, and 55.5 ppm were associated with the chemical shifts of C_1, C_4–C_5, C_3, C_6, and C_2 of D-glucosamine units in the CS, respectively. Both of relatively less intense peaks at 22.3 and 173.8 ppm were due to the chemical shift of methyl and carbonyl carbons from the residual acetyl groups of CS, respectively. In the case of lactose, the signals at 104.5, 73.5–79.5, 69.5–71.8, and 61.1 ppm were attributed to C_1, C_2, C_3–C_5, and C_6 of its ring unit, respectively. As compared to those of CS, the peak intensity and chemical shift of RP were changed because of the different chemical environment between them. At the chemical shift ranging from 58.35 to 106.44 ppm, the peaks of ^{13}C atoms associated with D-glucosamine units in the CS molecules were also observed for RP. The results indicated that skeleton structure of CS was almost preserved after the Millard reaction. In the case of the RP, the grafting of lactose units onto CS chains was evidenced by the increased signal intensity at ca. 180 ppm associated with the carboxyl carbons.

FT-IR spectra are a useful tool to indentify molecule structure after chemical modification. Fig. 3 shows FT-IR spectra of CS, lactose, and RP. For the lactose, the band at 1651 cm^{-1} evidenced the presence of carbonyl groups and the absorption at 3369 cm^{-1} resulted from the stretching vibration of O—H. A strong and broad band in the region of 3300–3500 cm^{-1} for the CS and RP was attributed to the overlapping between N—H and O—H stretching vibrations. The band of RP was lower than that of CS (3380 vs. 3374 cm^{-1}), possibly indicating the disruption of hydrogen bonding in the CS after the Millard reaction. As compared to that of CS, the C—H stretching vibration of RP at 2800–3000 cm^{-1} as well as CH_3 deformation and C—H bending at 1300–1500 cm^{-1} appeared more intense. The peaks at 1656 cm^{-1} and 1601 cm^{-1} were attributed

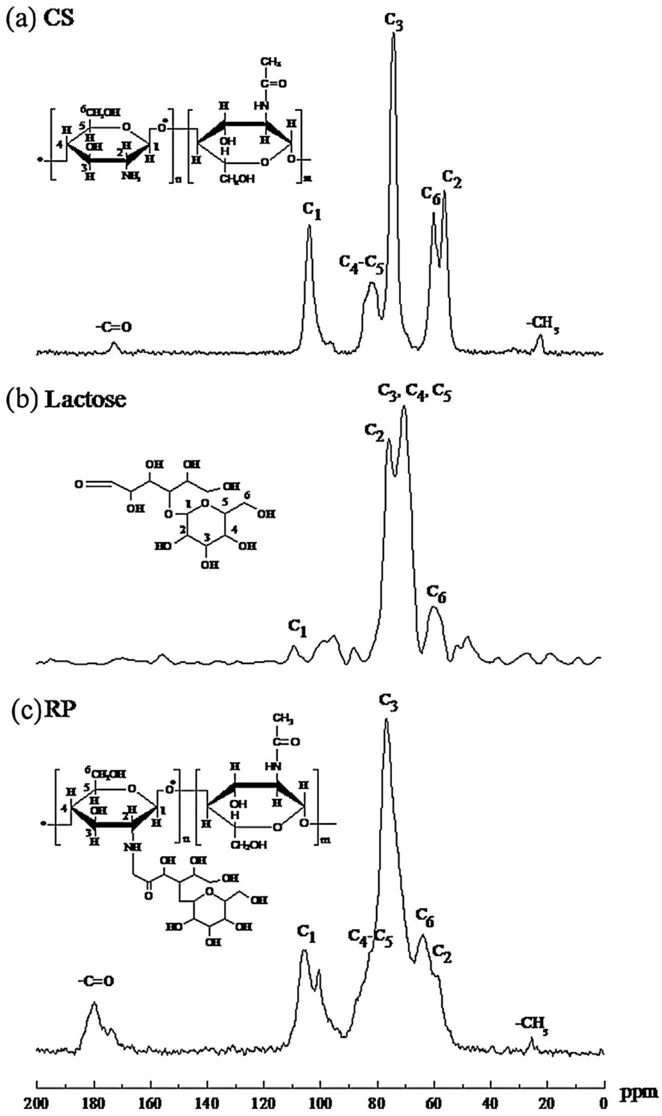

Fig. 2. CP-MAS ^{13}C-NMR spectra of CS (a), lactose (b), and RP (c).

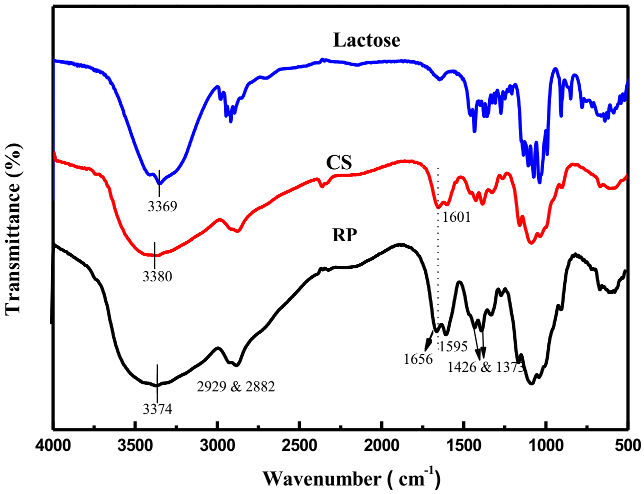

Fig. 3. FT-IR spectra of CS, lactose, and RP.

to the vibration of amide-I and amide-II of CS, respectively. It was noticed that both band of the RP became more intense than CS. And the amide-II vibration related to N—H bending shifted toward a lower frequency (~1595 cm^{-1}) for the RP, suggesting restricted mobility of N—H bending. In the view of these results above, one can conclude that lactose chains were successfully attached onto the backbones of CS via Millard reaction.

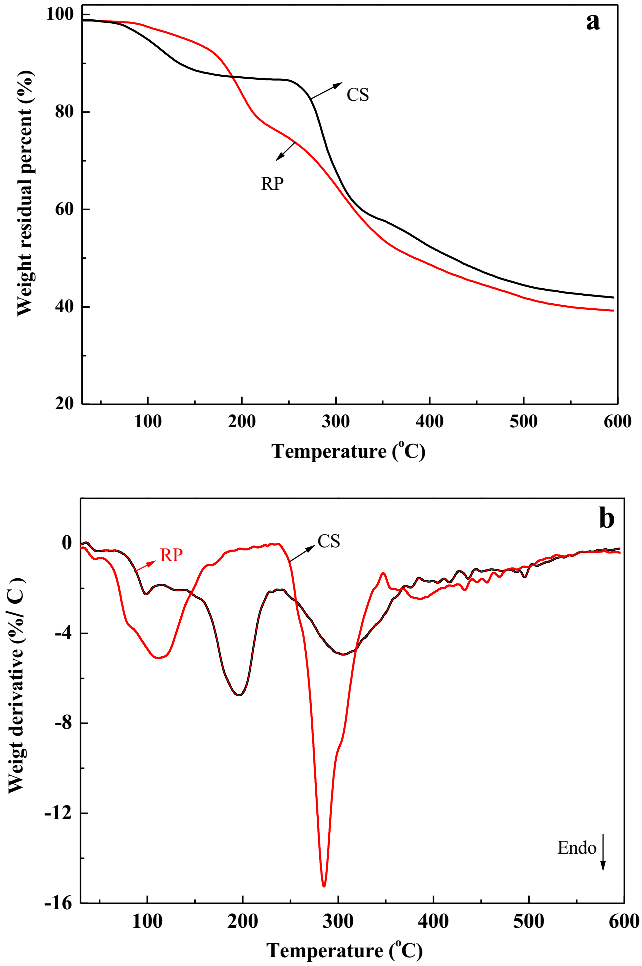

Fig. 4. Weight residual (a) and differential weight loss (b) curves of CS and RP as a function of temperature.

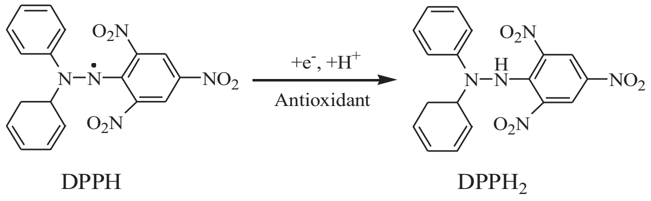

Fig. 5. Scavenging principle of DPPH radicals.

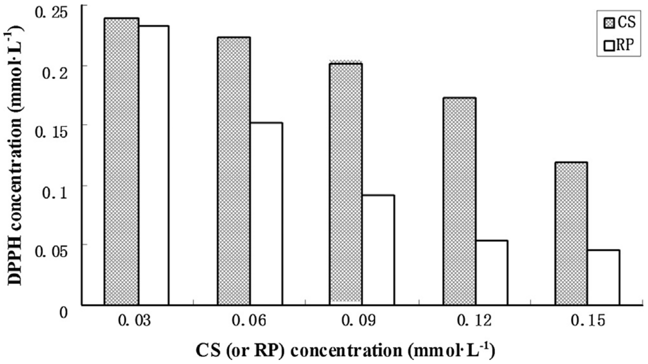

Fig. 6. Equilibrium concentration of DPPH radical vs. concentration of CS or RP.

Thermal stability may be another concern of anti-oxidant in practical application. Fig. 4 presents weight residual percent (TG) and differential weight loss (DTG) curves of CS and RP, respectively. CS showed two steps of weight loss with elevating temperature. The first step lied between 35 and 283 °C. A small weight loss observed 110 °C is related to the release of crystal water and the melting of amorphous structure of CS with weight loss of 15%. The temperature at the maximum decomposition rate of CS was 283 °C. Three weight loss steps during the heating process occurred for RP at 100 °C, 195 °C, and 300 °C, respectively. The loss of crystal water of RP was located at the first step (~100 °C), which was lower than that of CS. The second and thirds steps of DTG curves could be caused by its thermal degradation, and its maximum weight loss rate occurred at 195 °C, which also indicated inferior thermal stability of RP with respect to CS.

3.3. Radical-scavenging activity

The anti-oxidant capacity of RP was evaluated by their scavenging effect of 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Hatano et al.,

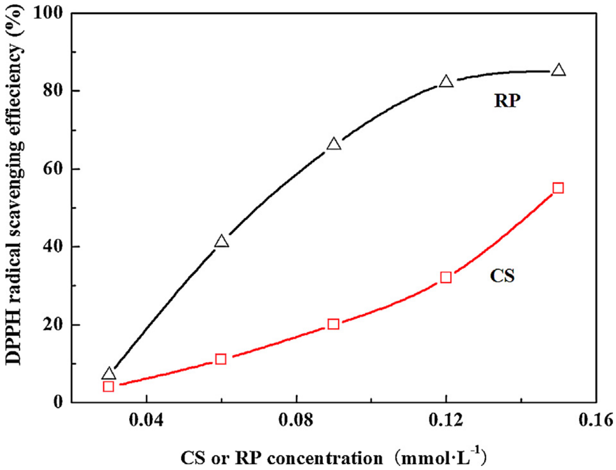

Fig. 7. DPPH radical scavenging efficiency of CS or RP as a function of concentration.

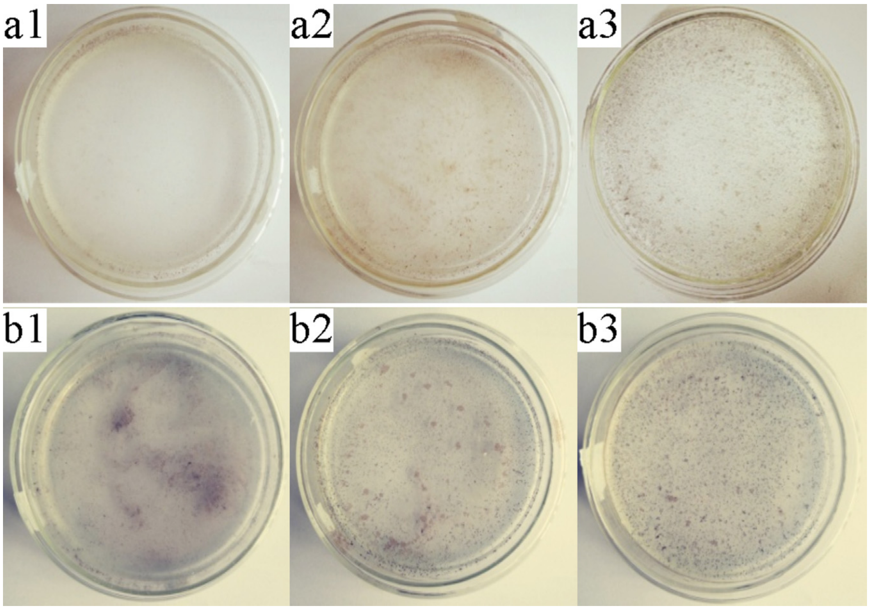

Fig. 8. The agar colony photos of the CS (top) and RP (bottom) observed at different time intervals.

1989). DPPH is a stable nitrogen-centered radical with the maximum absorption at the wavelength of 517 nm. When a radical scavenger was added, the lone electrons of DPPH molecules were paired and its scavenging principle of DPPH radical is illustrated in Fig. 5.

Fig. 6 shows the equilibrium concentration of DPPH radicals under varying concentration of CS or RP as a radical scavenger. With increasing concentration of CS or RP, the concentration of DPPH radical gradually decreased, suggesting that both CS and RP had a certain scavenging activity on the DPPH radical. But the clearance role of RP on DPPH radical appeared more pronounced than CS. Fig. 7 further presents the DPPH radical scavenging efficiency of CS or RP. Since CS contained amino–hydrogen atoms and hydroxyl–hydrogen atoms that would combine with nitrogen atoms of DPPH, the pairing of lone electrons occurred to eliminate the DPPH radical. Noteworthy, although the number of reactive amino group of RP decreased due to the Maillard reaction of CS with lactose, the radical scavenging efficiency of RP was higher than that of CS and the maximum efficiency of RP reached 85%. Rao et al. (2011) prepared the Millard reaction product (CGMRP) from chitosan and glucose through the gamma irradiation-induced method and investigated its anti-oxidant activity. It was found that the scavenging effect of CGMRPs on DPPH increased with increasing the concentration of DPPH. As a result, CGMRPs formed at 100 kGy dose showed 89% DPPH radical scavenging, which was comparable to that of RP in the present study. Dong et al. (2014) studied the influence of reaction conditions on anti-oxidant property of chitosan-fructose Maillard reaction product (CFMRP). It was shown that the maximum DPPH radical scavenging efficiency of 91.45% was achieved for CFMRP. Therefore, compared to the above reported Maillard reaction product from chitosan and mono-saccharides, RP derived from CS and lactose in this work exhibited the comparable radical scavenging efficiency, both of which exceeded 80% at the high concentration of DPPH.

3.4. Biodegradation test

Biodegradation is an important property of biobased materials for many practical applications, and so the biological degradation ability of RP was evaluated through the *Agar* colony degradation test with CS or RP samples as the sole carbon source. Fig. 8 shows the growth status photos of *Aspergillus niger* colony on the samples over a period of 1 week (a1 and b1), 2 weeks (a2 and b2), and 3 weeks (a3 and b3), respectively. It was clearly showed that the growth of colony in RP medium appeared more flourishing than CS, which evidenced better biodegradability of RP than CS.

4. Conclusions

In this study, the novel recombinant polysaccharide (RP) based on polysaccharide-disaccharide were successfully synthesized from lactose and oligo-chitosan using Maillard reaction. The chemical structure of RP was confirmed in terms of FT-IR and CP/MAS ^{13}C-NMR. TGA results showed that thermal stability of RP was found to be inferior to that CS. It was found that DPPH free radical scavenging ability of RP showed superior oxidation resistance to CS, which indicated that antioxidant properties of CS were remarkably promoted by Maillard reaction. In addition, the *Agar* colony degradation experiment demonstrated that better biodegradability of RP was observed as compared to CS. The findings would provide a novel route for the design and preparation of antioxidant RP using Maillard reaction.

Acknowledgments

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