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Oxidation of β-glucan extracted from *Poria Cocos* and its physiological activities

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ABSTRACT

Water-insoluble (1-3)-β-D-glucan isolated from the sclerotium of *P. cocos* exhibits little physiological activity. Therefore, it is advantageous to produce a value-added product from *P. cocos*. Extracted from the sclerotium of *P. cocos*, the (1-3)-β-D-glucan was oxidized by using TEMPO/NaBr/NaClO oxidation system and the water-soluble oxidized product was prepared. The structural and physiological properties of the derivative were investigated. The composition of the oxidized product was confirmed by Fourier transform infrared spectroscopy, the molecular weight parameters were obtained by LLS analysis. The oxidation caused the enhancement of *in vitro* bile acid binding capacity of the polysaccharides which would be explained by the improved water solubility and structural changes caused by oxidation. In addition, *in vitro* hydroxyl radical scavenging activity of the derivative was observed.

1. Introduction

Recently, the fungal polysaccharides as a functional food and a source for the development of biomedical drugs have attracted much attention. *P. cocos* (Fuling), a fungus that grows on the roots of pine trees, is one of the most important traditional medicines in China and other Asian countries, and has many culinary and medical uses such as anti-inflammatory, antitumor, complement activating, and immune stimulating activities (Kanayama, Adachi, & Togami, 1983; Lee et al., 2004; Yasukawa et al., 1998; Yu & Tseng, 1996). The main chemical component termed pachyman of *P. cocos* sclerotium is a water-insoluble (1-3)-β-D-glucan, which exhibits little physiological activity (Wang, Zhang, Li, Hou, & Zeng, 2004). To extend the use of pachyman, derivatizations such as partial carboxymethylation and sulfation of hydroxyl groups of pachyman have thus been prepared to increase their physiological activities with increasing water-solubility (Wang, Zhang, Ruan, et al., 2004; Wang, Yu, & Mao, 2009). However, these approaches for the carboxymethylation and sulfation of hydroxyl groups are undesirable, because virulent reagents such as chloroacetic acid and chlorosulfonic acid were used as reactants, respectively.

Recently, water-insoluble β-cyclodextrin, chitins, regenerated celluloses, paramylon and curdlan become water-soluble by the TEMPO/NaBr/NaClO oxidation system through partial or complete conversion of the C6 primary hydroxyls to carboxylate groups, producing a high-yield of polyuronic acids, and this TEMPO-mediated oxidation has pioneered a new field of carbohydrate chemistry (Fraschini & Vignon, 2000; Isogai & Kato, 1998; Kato, Kaminaga, Matsuo, & Isogai, 2004; Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999; Tamura, Wada, & Isogai, 2009).

Therefore, in this study, in order to obtain water-soluble polysaccharides, the water-insoluble ones extracted from the sclerotium of *P. cocos* were subjected to oxidation by the TEMPO/NaBr/NaClO oxidation system, the structure of the derivative, the changes in the water solubility and molecular weight were then investigated. Moreover, *in vitro* bile acid binding and antiradical capacities of the derivative were evaluated for the first time.

2. Materials and methods

2.1. Materials

On the basis of the previous method (Wang, Cheng, Mao, Fan, & Wu, 2009), polysaccharides were extracted from the sclerotium of *P. cocos* that was purchased from a local market. The sclerotium powder was defatted and extracted in water to remove water-soluble polysaccharides. Then the resulting residue was immersed in aqueous NaOH and extracted with the assistance of ultrasonication. The extracted liquid fraction was collected, decolorized and deproteinated, then dialyzed (regenerated cellulose tubing; Mw cut-off 5000) against tap water and distilled water for several days and finally lyophilized (Labconco, U.S.A.) to obtain a white powder. TEMPO, sodium bromide, 12% sodium hypochlorite solution, and other reagents and solvents were of analytical grade, and used

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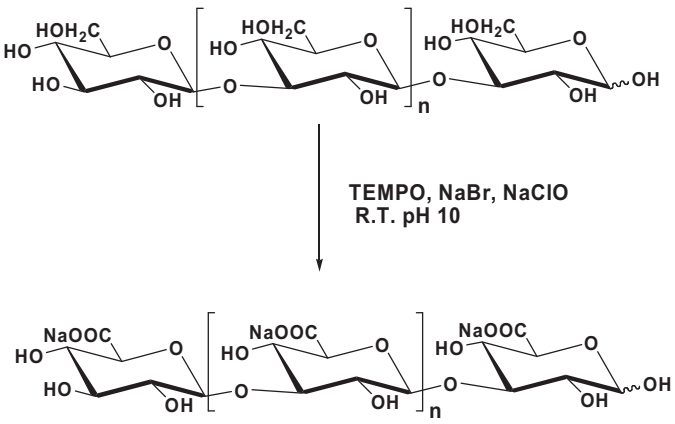

Fig. 1. TEMPO-mediated oxidation of (1-3)-β-D-glucan to prepared water-soluble(1-3)-β-D -polyglucuronic acid sodium salt.

without further purification. Distilled water of HPLC grade was purchased from Guangzhou Watsons Water Co., Ltd.

2.2. Preparation of oxidized derivatives

It was carried out by modifying the method of Isogai (Isogai & Kato, 1998). pachyman (1 g) was suspended in water (100 mL) at pH 10 containing TEMPO (0.016 g, 0.1 mmol) and sodium bromide (0.1 g, 1 mmol), and the suspension was stirred at room temperature. TEMPO-mediated oxidation was started by adding 9.3g of the 12% NaClO solution. The pH of the mixture was maintained to be 10 by adding 0.5 M NaOH until no NaOH consumption was observed. Then, the mixture was dialyzed with de-ionized water, and freeze-dried. Yields of the oxidized products, which were calculated based on their chemical structures, were about 85.4% (Fig. 1).

2.3. FT-IR analyses

Fourier transform infrared (FT-IR) spectra was obtained by using a Nicolet FT-IR spectrometer (Magna-IR 760 E.S.P, Nicolet Instrument Corp., Madison, WI). Samples were ground with potassium bromide (KBr) at a ratio of 1:20 and pressed into a thin pellet for FT-IR analysis.

2.4. LLS measurement

The TEMPO-oxidized product (1 mg) was dissolved in 0.2 M NaCl (1 mL), and optical clarification was achieved by filtration through a sand filter followed by a 0.2 μm pore-size filter (Whatman, England) into the scattering cell (Fused Silica). Molecular mass parameters of the oxidized products were measured with a eight-angle laser light scattering instrument equipped with a He-Ne laser (λ = 658nm; HELEOS 8, Wyatt Technology Co., USA) at 25 °C. The value of 0.125 mL/g was used as the specific refractive index increment (dn/dc) of the oxidized product in 0.2 M NaCl (Tamura et al., 2009). Data acquisition and processing was performed with the ASTRA software (Wyatt Technologies).

2.5. Water solubility analyses

To investigate the water solubility of the derivative (Chang, Lee, Yoo, & Hyeon, 2006), the sample (3.0 g) was suspended in distilled water (5.0 mL) and the suspension was agitated at 25 °C for 24 h. After centrifugation at 1600 g for 15 min, the collected supernatant (2.0 mL) was mixed with three volumes of ethanol. The precipitates were recovered by centrifugation at 3500 g for 15 min, vacuum-dried at 40°C, and weighed.

Table 1
Experimental results from LLS for the native and oxidized *P. cocos* polysaccharides.

Sample	Solvent	$<S^2>^{1/2}$ (nm)	$M_w \times 10^4$ (g/mol)
Native	Me_2SO	56.4	12.3
Oxidized	0.2 M NaCl	37.7	9.06

2.6. Bile acid binding capacity

Based on the method of Boyd, Eastwood, and MacLean (1966) and Camire, Zhao, and Violette (1993), the effect of the oxidation on the *in vitro* bile acid capacity of the polysaccharides was investigated. After samples were added to 0.01 M sodium phosphate buffer (pH 7.0) containing 500 μM bile acid to yield 2.5 mg/mL, they were treated at 37 °C for 2 h and then filtered. The resulting samples (1.0 mL) were treated with 70% sulfuric acid (5.0 mL) for 5 min, and then 0.25% furfural (1.0 mL) was added. After 1 h, absorbance was measured at 510 nm. The phosphate buffer without bile acid was used for a reagent blank. The levels of unbound bile acid were obtained using a standard curve prepared with pure bile acid.

2.7. HO• scavenging activity

Hydroxyl radical scavenging activity of sample was measured using a modified Cumbes and Smironoff'method (Cumbes & Smironoff, 1989). The reaction mixture, containing different samples (0.20–10.0 mg/mL), was incubated with 11.0 mM EDTA–Fe (2.0 mL), 0.2% H_2O_2(2 mL), and 20.0 mM sodium salicylate (1.0 mL) in 5.0 mL sodium phosphate buffer (pH 7.4) for1h at 37°C, and hydroxyl radical was detected by monitoring absorbance at 520 nm. In the control, sample was substituted with distilled water, and sodium phosphate buffer replaced H_2O_2. The capability of scavenging to hydroxyl radical was calculated using the following equation:

$$\text{scavenging effect}(\%) = (1 - A_{\text{sample}\,520}/A_{\text{control}\,520}) \times 100\%$$

3. Result and discussion

3.1. Structural and molecular weight analyses of the TEMPO-oxidized products

Fig. 2 displays the FT-IR spectrum of oxidized polysaccharides which was compared with that of native one. After oxidation, two new bands at 1619 and 1419 cm^{-1} were observed, which were characteristic of the stretching vibration of asymmetric and symmetric carboxyl groups, respectively (Jin, Zhang, Yin, & Nishinari, 2006; Yang & Du, 2003). Therefore, the FT-IR showed the existence of carboxyl groups in the derivative.

The TEMPO-oxidized products prepared from pachyman with the NaClO addition level of 15 mmol/g were analyzed by LLS to determine their molecular weight parameters. The weight average values of pachyman and oxidized products which are listed in Table 1 were 12.3×10^4 and 9.06×10^4, respectively. Thus, substantial depolymerization occurred as a result of TEMPO-oxidization.

3.2. Water solubility and bile acid binding capacity

Table 2 shows the water solubility and molecular mass of the derivative. Compared with the native sample, the introduction of carboxyl groups improved the water solubility of *P. cocos* polysaccharides, it would be explained by elevated hydrophilic properties of the derivative due to oxidation, making it more soluble. The increased solubility as a result of oxidation has been observed in the literature (Tamura et al., 2009).

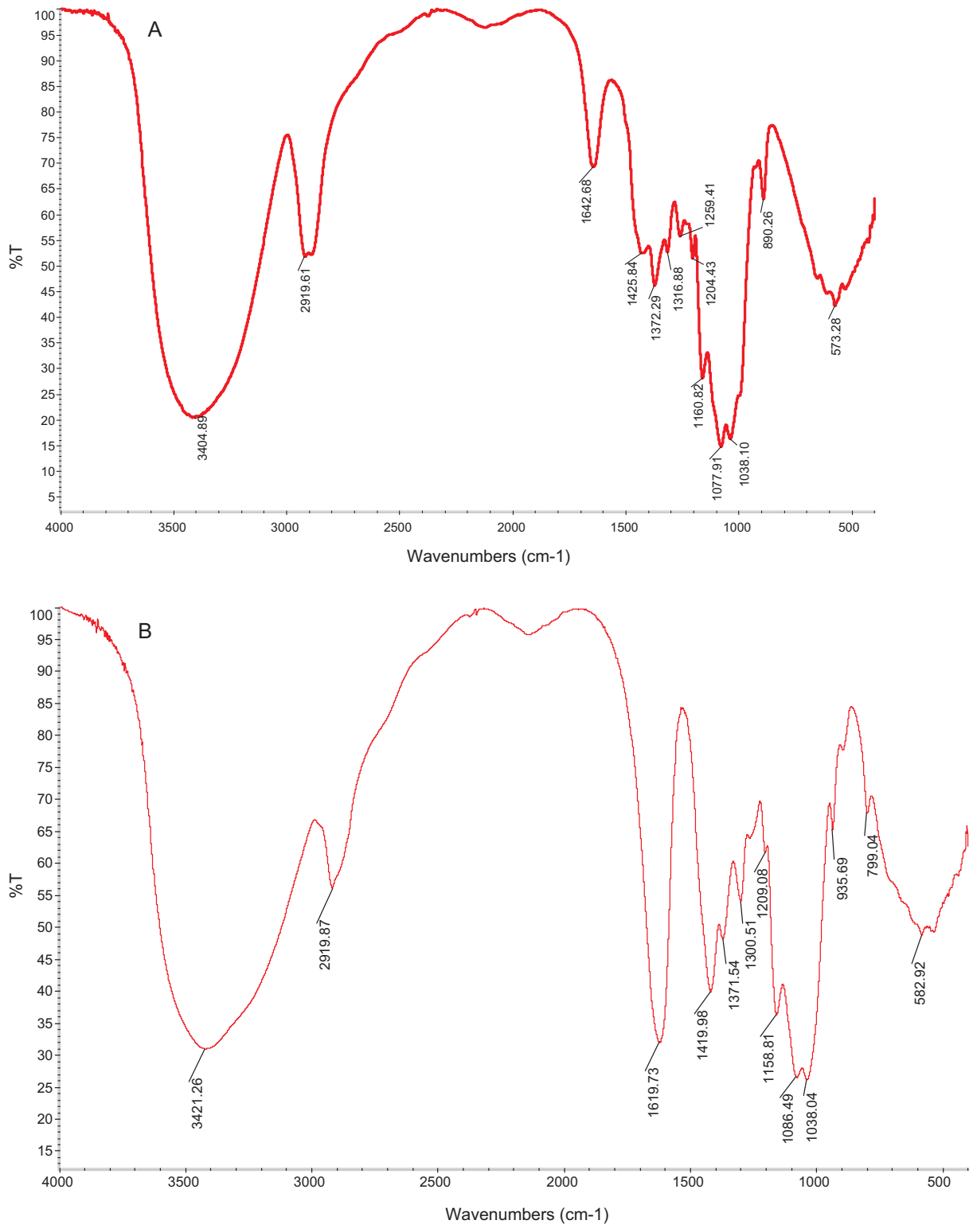

Fig. 2. FT-IR spectrum of native (A) and oxidized (B) polysaccharides extracted from *P.cocos*.

Table 2
Water solubility and bile acid binding capacity of native and oxidized *P. cocos* polysaccharides.

Sample	Water solubility (%)	Bile acid binding (μM/mg, dry matter)
Native	0	9.31
Oxidized	95.7	15.25

Binding of bile acids and subsequent excretion in feces have been recognized as a significant mechanism to eliminate excess cholesterol because bile acids are steroid carboxylic acids synthesized in liver from cholesterol (Yang & Du, 2003; Zhang, Zhang & Cheung, 2003; Parvathy, Susheelamma, Tharanathan & Gaonkar, 2005). Therefore, high binding capacity of bile acids suggests a possible ability to lower cholesterol in the body. It is well-known that

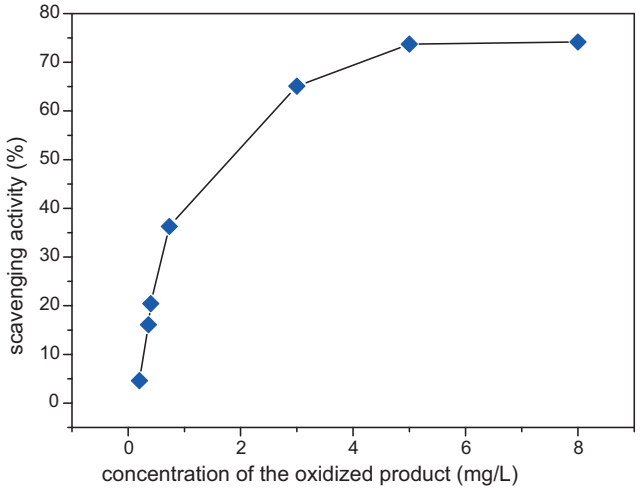

Fig. 3. Scavenging effect of oxidized product on hydroxyl radicals.

β-glucan reduces blood cholesterol levels, one of the major mechanisms of the cholesterol-lowering activity of β-glucan is that it increases the intestinal viscosity and decreases the absorption of cholesterol and bile acids in the body, consequently promoting their excretion (Zhang et al., 2003).

The bile acid binding capacities of native and oxidized polysaccharides were also presented in Table 2. The results showed that both polysaccharides had bile acid binding activity. One striking feature is that there was significantly higher binding capacity of bile acids with the derivative than with the native polysaccharides, implying more cholesterol-lowering effects. It is reported that water-soluble dietary fibers are more effective in lowering cholesterol levels than water-insoluble dietary fibers (Brown, Rosner, Willett, & Sacks, 1999; Liu et al., 2002). Water solubility also appears to be involved in biological activities such as bile acid binding capacity (Yoo et al., 2005). Therefore, better functionality of oxidized polysaccharides to bind bile acids *in vitro* might be partly explained by their improved water solubility.

3.3. Scavenging effect of oxidized product on hydroxyl radicals

HO• is the most reactive oxygen species and can easily penetrate through cell membranes. HO• can react with important biomolecules including carbohydrates, proteins, lipids, and DNA in cells, causing tissue damage or cell death. Removing HO• is very important for protection of biological systems (Yuan, Zhang, Fan, & Yang, 2008). As shown in Fig. 3, the oxidized product was found to possess the HO• scavenging activity, which increased as its concentration increased to certain extent and then appeared to reach a plateau. Compared with the derivative, the native polysaccharides showed no antioxidant activity. It was reported that the antioxidative capacities of sulfate of low-molecular-weight agar (LMAG) may be due to scavenging free radicals by electron-transfer (ET) to form stable macromolecular radicals through the sulfate group (Chen, Tsai, Huang, & Chen, 2009), so we think that the antioxidant activity presented by the oxidized product is in the same way as the ET from the carboxylate group of the modified polymer to the free radical (R•) to form a stable free-radical ion. The oxidized product loses an electron from carboxylate groups to form stable radicals.

4. Conclusions

Pachyman was subjected to oxidation and the physiological properties of the resulting derivative were investigated. The results demonstrated that water solubility, *in vitro* bile acid binding capacity and antioxidant activity of oxidized polysaccharides was enhanced by introducing carboxylate groups into the pachyman structure. However, significant depolymerization occurs on the main chains during the oxidation. The oxidized polysaccharides would be positively expected to have several improved health benefits including reduction of cholesterol and blood pressure.

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