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Carboxymethylated β-Glucan Derived from *Poria cocos* with Biological Activities

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Water-insoluble β-(1−3)-D-glucan isolated from the sclerotium of *Poria cocos* hardly exhibits biological activity. Therefore, it is advantageous to produce a value-added product from *P. cocos*. We extracted the β-(1−3)-D-glucan from the sclerotium of *P. cocos* and synthesized a carboxymethylated derivative. The structural and physiological properties of the derivative were investigated. The carboxymethylation of the polysaccharides was confirmed by Fourier transform infrared spectroscopy, and the degree of substitution (DS) and molecular weight were obtained by the potentiometric titration and gel permeation chromatography (GPC) analysis, respectively. The carboxymethylation 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 carboxymethylation. In addition, *in vitro* antiradical capacity of the derivative was observed by the method of 2,2-diphenyl-1-picrylhydrazyl (DPPH).

KEYWORDS: *Poria cocos*; carboxymethylation; bile acid; DPPH

INTRODUCTION

Poria cocos (Fu Ling), 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 (*1*−*4*). The main component termed pachyman of *P. cocos* sclerotium is a water-insoluble β-(1−3)-D-glucan, which hardly exhibits biological activity (*5*).

To extend the use of polysaccharides, various chemical modifications, such as sulfation, methylation, and carboxymethylation, have been widely carried out and their derivatives have been intensively studied because of industrial as well as scientific interests. Especially, carboxymethylation has often been applied to impart better water solubility to polysaccharides (*6*, *7*). When hydroxyl groups of polysaccharides are etherified with carboxymethyl groups, the hydrophilicity of the polysaccharides is enhanced. Therefore, the improved water solubility provides better functional attributes (*8*). However, few studies on the biological activities of the carboxymethylated polysaccharides extracted from the sclerotium of *P. cocos* are available.

Therefore, in this study, the water-insoluble polysaccharides extracted from the sclerotium of *P. cocos* were subjected to carboxymethylation and the structure of the derivative was characterized. 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.

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MATERIALS AND METHODS

Materials. On the basis of the previous method (*9*), polysaccharides were extracted from the sclerotium of *P. cocos* that was purchased from a local market. The powder of the sclerotium 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 ultrasonics. The extracted liquid fraction was collected, decolorized, deproteinated, then dialyzed (regenerated cellulose tubing; M_w cutoff of 5000) against tap water and distilled water for several days, and finally, lyophilized (Labconco, Kansas City, MO), to obtain a white powder.

Preparation of Carboxymethylated Derivatives. It was carried out by modifying the method of Shi et al. (*10*). With the assistance of ultrasonics (300 W), 2.0 g of polysaccharides was suspended in 4.3 mL of ethanol/water (80%, v/v), then 0.63 g of sodium hydroxide was added, and the system was subjected to a continuous reaction for 30 min at room temperature. A total of 2.0 g of monochloroacetic acid (MCA) in 4.3 mL of ethanol/water (80%, v/v) was added, and then another 0.63 g of sodium hydroxide in 4.3 mL of ethanol/water (80%, v/v) was added again up to a [NaOH]/[MCA] ratio of 1.5. After 1 h of reaction at 45 °C, the mixture was cooled to room temperature and neutralized with glacial acetic acid. The reaction product was precipitated in ethanol, filtered, purified, and dried under vacuum at 40 °C for 2 h prior to analyses.

Structural and Physicochemical Characterization. Fourier transform infrared (FTIR) spectra of the derivative was obtained using a Nicolet FTIR spectrometer (Magna-IR 760 ESP, 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 FTIR analysis.

The degree of substitution (DS) of the derivative was estimated from the acidimetric titration. The derivative (4.0 g) was heated at 700 °C for 30 min, and then the leftover was dissolved with purified water and moved to a beaker. After adding three drops of methyl red indicator, the solution was titrated with H_2SO_4 (0.05 mol/L) until the indicator turned to red and

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then another 10.0 mL of H_2SO_4 (0.05 mol/L) was added. The solution was then boiled for 10 min, followed by titration with a solution of NaOH (0.1 mol/L), until the color changing back to yellow. The degree of substitution was calculated on the basis of the following equation (*11*), where C is the milliequivalent of acid consumed per gram of sample, A (in milliliters) is the volume of H_2SO_4 used for titration, B (in milliliters) is the volume of NaOH added, and W (in grams) is the sample mass:

$$DS = 0.162C/(1 - 0.08C)$$

$$C = (2 \times 0.05A - 0.1B)/W$$

Molecular masses of the derivatized samples were determined by gel permeation chromatography (GPC) (SDS 9414i, Schambeck SFD GmbH, Germany). The GPC system was connected with a refractive index detector (Schambeck SFD GmbH, Germany). Deionized water was used as an eluent, and the flow rate was 0.5 mL/min.

To investigate the water solubility of the derivative (*12*), 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.

Bile Acid Binding Capacity. On the basis of the method by Boyd et al. (*13*) and Camire et al. (*14*) the effect of carboxymethylation 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 250 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 25% furfural (1.0 mL) was added. After 1 h, absorbance was measured at 510 nm.

Scavenging Activity. The free-radical-scavenging activity of the carboxymethylated derivative was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (*15*). An aliquot of 4.5 mL of DPPH solution (11.2×10^{-5} mg/mL) in methanol was added to 0.5 mL of sample at various concentrations, and methanol was used as a control. The reaction mixture was stirred, and its absorbance at 515 nm was measured after 30 min. The DPPH radical-scavenging activity (%) was calculated by the following equation:

$$\text{scavenging activity (\%)} = (1 - \text{absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}}) \times 100$$

Statistical Analysis. All experiments were carried out in triplicate. Statistical analysis was performed with one-way analysis of variance to decide a significance of difference among samples at the level of 5%.

RESULTS AND DISCUSSION

Figure 1 displays the FTIR spectrum of carboxymethylated polysaccharides, which was compared to that of the native one. After carboxymethylation, two new bands at 1603 and 1424 cm^{-1} were observed, which were characteristic of the stretching vibration of asymmetric and symmetric carboxyl groups, respectively (*7*, *16*). Moreover, the absorption band at 2918 cm^{-1} became prominent, which was attributed to the stretching vibration of methylene group (*17*). Therefore, the FTIR showed the existence of carboxymethyl groups in the derivative.

The molecular mass of the derivatives with different DS were investigated (**Table 1**). It is interesting to note that the molecular mass of the carboxymethylated derivatives increased from 3440 to 3990 kDa as the DS of the derivatives increased from 0.26 to 0.91. It implies the efficient substitution of hydroxyl groups in the polysaccharides by carboxymethyl groups with little degradation. The molecular mass increase of polymers after carboxymethylation has been observed by previous studies (*8*, *17*).

Table 2 shows the water solubility and bile acid binding capacity of the derivative compared to those of the native sample. The introduction of carboxymethyl groups improved the water solubility of *P. cocos* polysaccharides. It would be explained by

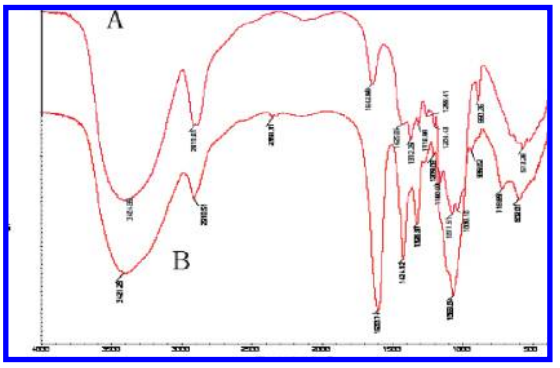

Figure 1. FTIR spectrum of (A) native and (B) carboxymethylated polysaccharides extracted from *P. cocos*.

Table 1. Variation of Weight-Average Molecular Weight (M_w) of the Carboxymethylated Polysaccharides with DS[a]

DS	0.26 ± 0.07	0.59 ± 0.05	0.81 ± 0.05	0.91 ± 0.03
M_w (kDa)	3440 ± 73.44	3460 ± 61.07	3610 ± 84.15	3990 ± 70.04

[a] Values are means ± standard deviation (SD).

Table 2. Water Solubility and Bile Acid Binding Capacity of Native and Carboxymethylated *P. cocos* Polysaccharides[a]

sample	water solubility (%)	bile acid binding (μM/mg, dry matter)
native	0 b	9.31 ± 0.52 b
carboxymethylated	98.4 ± 2.62 a	19.72 ± 1.73 a

[a] Values are means ± SD. Values with different letters within the same column are significantly different at the α = 0.05 level by Duncan's multiple range test.

elevated hydrophilic properties of the derivative because of carboxymethylation, making it more soluble. The increased solubility by carboxymethylation has been commonly observed in the literature (*18*).

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 the liver from cholesterol (*16*−*18*). Therefore, a high binding capacity of bile acids suggests a possible ability to lower cholesterol in the body. It is well-known that 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 (*17*).

The bile acid binding capacities of native and carboxymethylated 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 a 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 (*19*, *20*). Water solubility also appears to be involved in biological activities, such as bile acid binding capacity (*21*). Therefore, better functionality of carboxymethylated polysaccharides to bind bile acids *in vitro* might be partly explained by their improved water solubility.

The DPPH radical-scavenging capacity has been widely used to measure antioxidant activity (*15*). As a rapid and simple method, it is based on the reduction of the stable radical DPPH to yellow-colored diphenylpicrylhydrazine in the presence of a

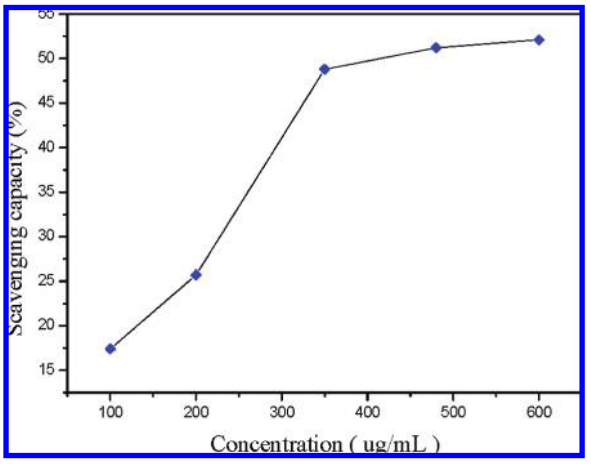

Figure 2. Effect of carboxymethylation of *P. cocos* polysaccharides on DPPH radical-scavenging activity.

hydrogen donor. The effect of carboxymethylation on the DPPH radical-scavenging activity of *P. cocos* polysaccharides was investigated as shown in **Figure 2**. The sample was found to possess the DPPH radical-scavenging activity, which increased as its concentration increased to a certain extent and then appeared to reach a plateau. In comparison to the derivative, the native polysaccharides showed no antioxidant activity.

In conclusion, a polysaccharide derivative was prepared from water-insoluble *P. cocos* polysaccharides by carboxymethylation, which imparted new physiological characteristics to the β-glucan. Our results revealed the increased solubility, *in vitro* bile acid binding capacity, and antioxidant activity of carboxymethylated polysaccharides compared to the native sample. Therefore, the carboxymethylated polysaccharides would be positively expected to have several improved health benefits, including a reduction of cholesterol and blood pressure.

Supporting Information Available: GPC chromatograms of carboxymethylated polysaccharides with various DS at 30 °C, a flow rate of 0.5 mL min^{-1}, and H_2O as the eluent. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Kanayama, H.; Adachi, N.; Togami, M. A new antitumor polysaccharide from the mycelia of *Poria cocos* Wolf. *Chem. Pharm. Bull.* **1983**, *31*, 1115–1118.

(2) Yu, S. J.; Tseng, J. Fu-Ling, a Chinese herbal drug, modulates cytokine secretion by human peripheral blood monocytes. *Int. J. Immunopharmacol.* **1996**, *18*, 37–44.

(3) Yasukawa, K.; Kaminaga, T.; Kitanaka, S.; Tai, T.; Nunoura, Y.; Natori, S. 3β-*p*-Hydroxybenzoyldehydrotumulosic acid from *Poria cocos*, and its anti-inflammatory effect. *Phytochemistry* **1998**, *48*, 1357–1360.

(4) Lee, K. Y.; You, H. J.; Jeong, H. G.; Kang, J. S.; Kim, H. M.; Dal Rhee, S. Polysaccharide isolated from *Poria cocos* sclerotium induces NF-κB/Rel activation and iNOS expression through the activation of p38 kinase in murine macrophages. *Int. Immunopharmacol.* **2004**, *4*, 1029–1038.

(5) Wang, Y. F.; Zhang, M.; Ruan, D.; Shashkov, A. S.; Kilcoyne, M.; Savage, A. V.; Zhang, L. N. Chemical components and molecular mass of six polysaccharides isolated from the sclerotium of *Poria cocos*. *Carbohydr. Res.* **2004**, *339*, 327–334.

(6) Lee, S.; Park, H.; Seo, D.; Choi, Y.; Jung, S. H. Synthesis and characterization of carboxy-methylated cyclosophoraose, and its inclusion complexation behavior. *Carbohydr. Res.* **2004**, *339*, 519–527.

(7) Jin, Y.; Zhang, H.; Yin, Y.; Nishinari, K. Comparison of curdlan and its carboxymethylated derivative by means of rheology, DSC, and AFM. *Carbohydr. Res.* **2006**, *341*, 90–99.

(8) Zhang, M.; Cheung, P. C. K.; Zhang, L.; Chiu, C.-M.; Ooi, V. E. C. Carboxymethylated β-glucans from mushroom sclerotium of *Pleurotus tuber-regium* as novel water-soluble anti-tumor agent. *Carbohydr. Polym.* **2004**, *57*, 319–325.

(9) Wang, Y. J.; Cheng, Z.; Mao, J. W.; Fan, M. E.; Wu, X. Q. Optimization of ultrasonic-assisted extraction process of *Poria cocos* polysaccharides by response surface methodology. *Carbohydr. Polym.* **2009**, *77*, 713–717.

(10) Shi, Q. D.; Jiang, X. M. Twice alkalization method for preparation of carboxymethyl-pacharaman. *Nat. Prod. Res. Dev.* **1996**, *8*, 78–81.

(11) Huang, C. H. Studies on the extraction methods and modification of polysaccharides from *Poria cocos*. M.S. Dissertation, Jinan University, Guangzhou, China, **2002**.

(12) Chang, Y. J.; Lee, S.; Yoo, M. Y.; Hyeon, G. L. Structural and biological characterization of sulfated-derivatized oat β-glucan. *J. Agric. Food Chem.* **2006**, *54*, 5815–3818.

(13) Boyd, G. S.; Eastwood, M. A.; MacLean, N. Bile acids in the rat: Studies in experimental occlusion of the bile duct. *J. Lipid Res.* **1966**, *7*, 83–87.

(14) Camire, M. E.; Zhao, J.; Violette, D. A. In vitro binding of bile acids by extruded potato peels. *J. Agric. Food Chem.* **1993**, *21*, 2391–2394.

(15) Kroyer, G.; Hegedus, N. Evaluation of bioactive properties of pollen extracts as functional dietary food supplement. *Innovative Food Sci. Emerging Technol.* **2001**, *2*, 171–174.

(16) Yang, J.; Du, Y. Chemical modification, characterization and bioactivity of Chinese lacquer polysaccharides from lac tree *Rhus vernicifera* against leukopenia induced by cyclo-phosphamide. *Carbohydr. Polym.* **2003**, *52*, 405–410.

(17) Zhang, M.; Zhang, L.; Cheung, P. C. K. Molecular mass and chain conformation of carboxy-methylated derivatives of β-glucan from sclerotia of *Pleurotus tuber-regium*. *Biopolymers* **2003**, *68*, 150–159.

(18) Parvathy, K. S.; Susheelamma, N. S.; Tharanathan, R. N.; Gaonkar, A. K. A simple non-aqueous method for carboxymethylation of galactomannans. *Carbohydr. Polym.* **2005**, *62*, 137–141.

(19) Brown, L.; Rosner, B.; Willett, W.; Sacks, F. Cholesterol-lowering effects of dietary fiber: A meta-analysis. *Am. J. Clin. Nutr.* **1999**, *69*, 30–42.

(20) Liu, S.; Buring, J.; Sesso, H.; Rimm, E.; Willett, W.; Manson, J. A prospective study of dietary fiber intake and risk of cardiovascular disease among women. *J. Am. Coll. Cardiol.* **2002**, *39*, 49–56.

(21) Yoo, S. H.; Lee, J. S.; Park, S. Y.; Kim, Y. S.; Chang, P. S.; Lee, H. G. Effects of selective oxidation of chitosan on physical and biological properties. *Int. J. Biol. Macromol.* **2005**, *35*, 27–31.

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