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Structural and Biological Study of Carboxymethylated *Phellinus linteus* Polysaccharides

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Polysaccharides isolated from *Phellinus linteus* were chemically modified by carboxymethylation, and the structural and physiological properties of the derivative were investigated. ^{13}C NMR spectroscopy showed that the polysaccharides extracted from *P. linteus* contained (1−3)-β-glucans with a (1−6)-linkage. The carboxymetehylation of the *P. linteus* polysaccharides was confirmed by Fourier transform infrared spectroscopy, and the degree of substitution was obtained by the potentiometric titration, which was calculated to be 0.63. The bronchoalveolar lavage experiments showed that the carboxymethylated derivative raised the nitric oxide production. In addition, the carboxymethylation stimulated in vitro cytotoxic activity against the HT1080 cell line. Thus, the derivative exhibited the enhanced activity of immune systems, which would be explained by the improved water solubility and structural changes by carboxymethylation. However, a slight decrease in the 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of the derivative was observed.

KEYWORDS: ***Phellinus linteus*; carboxymethylation; cytotoxic activity; nitric oxide; DPPH radical scavenging activity**

INTRODUCTION

Phellinus linteus, a mushroom, is a species of the genus *Phellinus*, which is commonly called "Sangwhang" in Korea and "Mesimakobu" in Japan. It is popular in northeastern Asian countries and has been traditionally used as food and medicine. Recently, *P. linteus* has received great attention mainly focused on its immunological responses. It is reported in the literature that *P. linteus* extracts enhance the activity of immune systems by stimulating B-lymphocytes, T-lymphocytes, and macrophages, consequently inhibiting tumor cell growth and metastasis without toxicity (*1*−*4*). In addition to the immunomodulating properties of *P. linteus*, it is known to have antiallergic, anti-inflammatory, and hypoglycemic effects (*5*−*7*). The active constituents that are involved in these effects are considered to be polysaccharides and/or proteoglycans in *P. linteus* (*8*).

To extend the use of polysaccharides, various chemical modifications such as amination, sulfation, and carboxymethylation have been widely carried out and their derivatives have been intensively studied due to industrial as well as scientific interests. Especially, carboxymethylation has often been applied to impart better water solubility to polysaccharides (*9*, *10*). By etherifying hydroxyl groups of polysaccharides with carboxymethyl groups, the hydrophilicity of the polysaccharides is enhanced. Therefore, the improved water solubility provides better functional attributes such as antitumor activities (*11*, *12*). However, few studies are available on the carboxymethylated polysaccharides extracted from different species of mushrooms (*12*), and even any chemical derivatizations of *P. linteus* polysaccharides have not yet been reported.

Therefore, in this study, the polysaccharides extracted from the fruit body of the mushroom *P. linteus* were subjected to carboxymethylation and the structure of the derivative was characterized. Then, its antioxidant, nitric oxide synthesis, and cytotoxic activities were assayed.

MATERIALS AND METHODS

Isolation of *P. linteus* Polysaccharides. On the basis of the previous method (*13*), polysaccharides were extracted from *P. linteus* that was purchased from a local market. The homogenized fruit body of *P. linteus* (200 g) was suspended in distilled water (4000 g) under agitation at 95 °C for 1 h and then filtered through Whatman #41 and #4 filter papers. This procedure was repeated three times to remove unnecessary debris. After the filtrate was concentrated in a rotary evaporator, it was mixed with three volumes of ethanol for 24 h at 4 °C, followed by centrifugation at 3200*g* for 30 min. The precipitates were then dialyzed against distilled water for 5 days and freeze-dried. The chemical structure of the isolated polysaccharides was investigated by using ^{13}C nuclear magnetic resonance spectroscopy (300 MHz, Varian Co., Palo Alto, CA).

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Preparation of Carboxymethylated Derivatives. According to the method of Bao et al. (*14*), the polysaccharides (1 g) extracted from *P. linteus* were mixed with 2-propanol (30 mL) and stirred for 30 min at room temperature. After NaOH (13%, 1.26 mL) was slowly added over a period of 15 min, it was vigorously stirred for an additional 90 min. Then, chloroacetic acid (15%, 1.26 mL) was added dropwise into the mixture, which was stirred at 50 °C for 3 h. After the resulting product was cooled and neutralized with HCl, it was dialyzed against distilled water for 2 days and freeze-dried.

Structural and Physicochemical Characterization. To characterize the structure of the derivative, Fourier transform infrared (FT-IR) spectra were 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.

The degree of substitution of the derivative was estimated from the potentiometric titration. The derivative (4 g) was dissolved in 75 mL of ethyl alcohol (95%), and 5 mL of HNO_3 was added, followed by agitation for 2 min. After it was boiled for 5 min, the mixture was stirred for 15 min and set aside for settlement. After filtration, the precipitate was washed with ethyl alcohol (80%), heated to 60 °C, and then washed with methanol, which was removed by drawing air through it. Finally, the precipitate was dried at 105 °C for 3 h.

The solution for the potentiometric titration was prepared by mixing the dried sample (1.5 g), distilled water (100 g), and sodium hydroxide solution (0.4 N, 25 mL). Then, it was boiled for 30 min, followed by titration with a solution of HCl (0.4 N). The degree of substitution was calculated based on the following equation (*15*).

$$\text{degree of substitution} = \frac{0.162 \times A}{(1 - 0.058 \times A)}$$

$$A = \frac{(BC - DE)}{F}$$

where A is the milliequivalent of acid consumed per gram of sample, B (mL) is the amount of NaOH solution added, C is the normality of the NaOH solultion, D (mL) is the amount of HCl solution used for titration, E is the normality of the HCl solution, and F (g) is the sample mass.

Molecular masses of the native and derivatized samples were determined by gel permeation chromatography (GPC) (LC-900, Japan Analytical Instrument, Tokyo, Japan). The GPC system was connected with three columns in series (Jaigel-W254, Jaigel-W-253, and Jaigel-W252, Japan Analytical Instrument) and a refractive index detector (RI-50, Japan Analytical Instrument). Deionized water was used as an eluent, and the flow rate was 3.5 mL/min.

To investigate the water solubility of the derivative (*16*), the sample (3 g) was suspended in distilled water (5 mL) and the suspension was agitated at 25 °C for 24 h. After centrifugation at 1600*g* for 15 min, the collected supernatant (2 mL) was mixed with three volumes of ethanol. The precipitates were recovered by centrifugation at 3500*g* for 15 min, vacuum-dried at 60 °C, and weighed.

Physiological Property Measurements. The in vitro cytotoxic activity against HT1080 human fibrosarcoma cell line (Korean Cell Line Bank, Seoul, Korea) was investigated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (M-5655, Sigma-Aldrich Chemical Co., Milwaukee, WI) (*17*). The cells were grown in RPMI1640 medium (LM 011-01, JBI Welgene Co., Daegu, Korea) supplemented with 10% fetal bovine serum and 100 unit/mL penicillin−streptomycin solution (P-4458, Sigma-Aldrich Chemical Co.), which was maintained at 37 °C in 5% CO_2 environment for 92 h. After the HT1080 cells at various concentrations were incubated with the MTT solution at 37 °C for 4 h, 150 mL of dimethyl sulfoxide (Sigma-Aldrich Chemical Co.) was added to dissolve formazan crystals, followed by agitation on a plate shaker for 15 min. The optical density of the formazan solutions was then measured on the multiwell enzyme-linked immunosorbent assay (ELISA) automatic spectrometer reader (ELx800UV, Bio-Tek Instrument Inc., Windoski, VT) at 540 nm.

To investigate the effect of carboxymethylation on nitric oxide production, bronchoalveolar lavage (BAL) cells (5.0×10^5 cells/mL) obtained by the method of Shin et al. (*18*) were suspended in Dulbecco's modified Eagle's medium (DMEM) (12-604F, Cambrex Bio Science Walkersville Inc., Walkersville, MD) with 10% fetal bovine serum and 100 unit/mL penicillin−streptomycin. The cells (1 mL) were then transferred to 12 well tissue culture plates (Greiner, Nrtingen, Germany) and incubated at 37 °C for 2 h in 5% CO_2. After the media were replaced with phenol red-free DMEM, the native and carboxymethylated samples (5, 10, 25, 50, and 100 μg/mL) were added. After incubation for an additional 24 h, the culture supernatant (100 μL) was mixed with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthylene diamine dihydrochloride/2.5% H_3PO_4) (G-4410, Sigma-Aldrich Chemical Co.) at room temperature for 2 min, and the absorbance was read at 540 nm (*19*). The amount of produced nitric oxide was obtained from the nitrite concentration, which was measured from a sodium nitrite standard curve.

The free radical scavenging activity of the carboxymethylated derivative was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (*20*). An aliquot of 2.7 mL of DPPH solution (6×10^{-5} M) in methanol was added to 0.3 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 differences among samples at the level of 5%. Duncan's multiple range test was then applied for mean comparisons.

Table 1. Structural Analysis of *P. linteus* Polysaccharides by ^{13}C NMR

chemical shift (δ)	C-1	C-2	C-3	C-4	C-5	C-6	C-6′
P. linteus polysaccharide	102.9	73.7	85.7	69.2	75.5	60.3	70.6
(1–6)-branched (1–3)-β-D-glucan (*21*, *22*)	103.0	72.8	86.2	68.4	76.3	60.9	70.0

RESULTS AND DISCUSSION

The structure of the polysaccharide isolated from *P. linteus* was studied by using ^{13}C NMR. **Table 1** shows the chemical shifts of *P. linteus* polysaccharides, which were attributed to carbons from C-1 to C-6 of (1−3)-β-D-glucan (*21*, *22*). In addition, another signal at δ70.6 was observed, which was a characteristic peak of a β-(1−6)-branch. It therefore indicated that the polysaccharides extracted from *P. linteus* contained (1−3)-β-glucans with a (1−6)-linkage.

The effect of carboxymethylation on the structure of *P. linteus* polysaccharides was investigated by FT-IR (**Figure 1**). After carboxymethylation, two new bands at 1610 and 1400 cm^{-1} were observed, which would be characteristic of the stretching vibration of asymmetric and symmetric carboxyl groups, respectively (*10*, *23*). Moreover, the absorption band at 2919 cm^{-1} became prominent, which was attributed to the stretching vibration of methylene group (*24*). Therefore, the FT-IR showed the existence of carboxymethyl groups in the derivative of which the degree of substitution was also determined by the method of potentiometric titration to be 0.63.

The molecular mass and water solubility of the derivative were investigated and compared with those of the underivatized sample (**Table 2**). It is interesting to note that the carboxymethylated derivative had a higher molecular mass than the underivatized sample. It implies the efficient substitution of hydroxyl groups in the polysaccharides by carboxymethyl

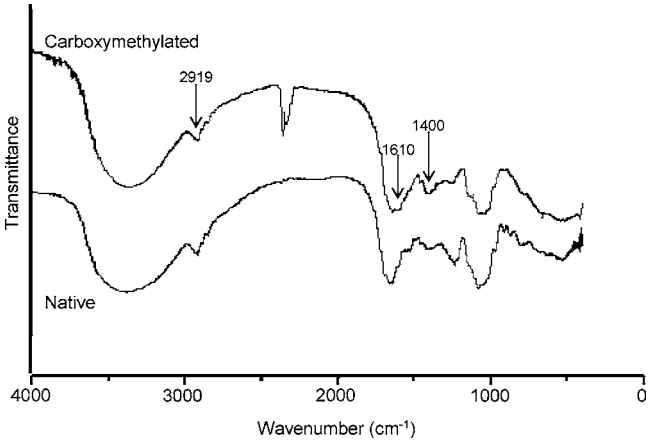

Figure 1. FT-IR spectrum of native and carboxymethylated polysaccharides extracted from *P. linteus*.

Table 2. Molecular Masses and Water Solubility of Native and Carboxymethylated *P. linteus* Polysaccharides[a]

sample	molecular mass (kDa)	water solubility (%)
native	860 b	73.3 b
carboxymethylated	1157 a	91.7 a

[a] Values with different letters within the same column are significantly different at the $\alpha = 0.05$ level by Duncan's multiple range test.

groups with little degradation. The molecular mass increase of polymers after carboxymethylation has been observed in the literature (*11*, *12*, *24*). **Table 2** also shows that the introduction of carboxymethyl groups improved the water solubility of *P. linteus* polysaccharides. It would be explained by elevated hydrophilic properties of the derivative due to carboxymethylation, making it more soluble. The increased solubility by carboxymethylation has been commonly observed in the literature (*25*).

Nitric oxide is a free radical gas that is generated from L-arginine by nitric oxide synthase enzymes. It is recognized that nitric oxide is involved in important physiological and pathological functions by acting as a regulator of blood pressure and a neurotransmitter in both central and peripheral nervous systems (*26*). Also, in the immune system, activated macrophages produce nitric oxide for the defense against infectious invaders (*27*).

It is reported that *P. linteus* induces the synthesis of nitric oxide (*3*). Even though the mechanisam of *P. linteus* to stimulate the nitric oxide production is not yet known, it might be related to β-glucan included in *P. linteus*. β-Glucan enhances the activity of the immune system by binding to specific receptors on macrophages (*28*−*30*). The recognition of β-glucan via the specific receptors activates macrophages, secreting various biologically active substances such as nitric oxide.

The effect of native and carboxymethylated *P. linteus* polysaccharides on nitric oxide production was investigated. It is shown in **Figure 2** that the carboxymethylated derivative generated more nitric oxide than the native sample at all concentrations tested in this study. Furthermore, the derivative induced the synthesis of nitric oxide in a concentration-dependent manner while the level of nitric oxide produced by the underivatized polysaccharide remained relatively constant. Especially, the amount of produced nitric oxide increased 1.8-fold at a concentration of 100 μg/mL.

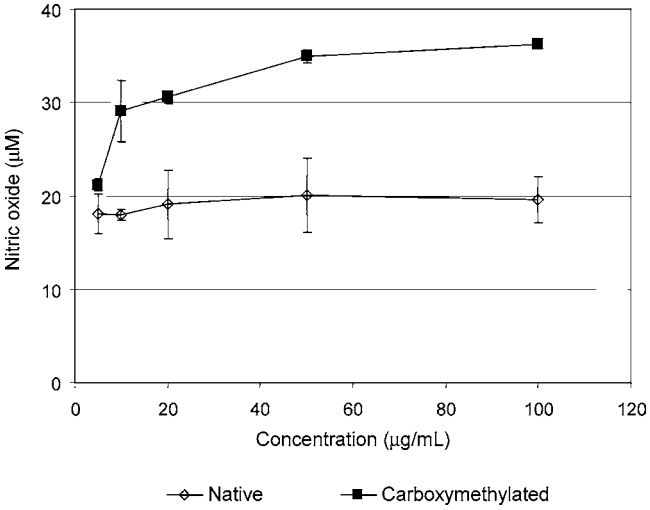

Figure 2. Production of nitric oxide by BAL cells treated with native and carboxymethylated *P. linteus* polysacchrides (the error bars indicate the standard deviation).

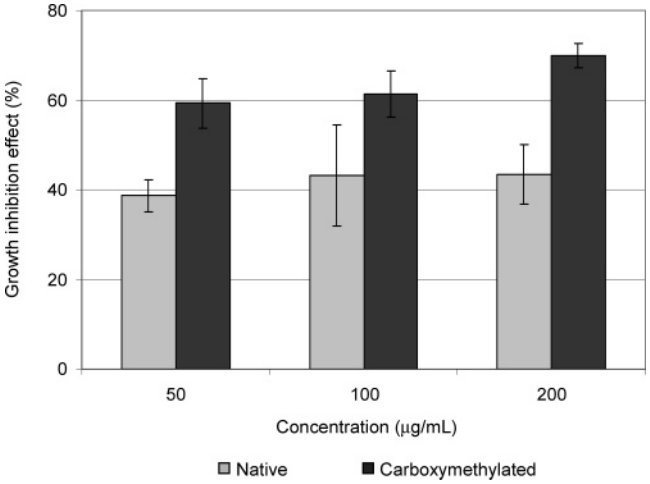

Figure 3. MTT assay of native and caboxymethylated *P. linteus* polysaccharides on HT1080 (human null fibrosarcoma) cells (the error bars indicate the standard deviation).

This enhanced nitric oxide synthesis of the derivative is possibly due to the introduction of carboxymethyl groups to the polymer chain. It is recognized that the improvement of water solubility is effective in enhancing the immunomodulatory effects of β-glucan (*31*). Therefore, the increased water solubility of the carboxymethylated derivative might contribute partly to its improved interaction with the specific receptors, effectively stimulating the activity of macrophages to produce nitric oxide.

The inhibition activities of native and carboxymethylated *P. linteus* polysaccharides against the growth of human fibrosarcoma HT1080 cells were assessed at three different concentrations (**Figure 3**). Even though both samples were shown to have inhibitory effects on the growth of HT1080 cells, no obvious concentration dependence was observed. However, when the cells were exposed to the derivative, more growth inhibition was observed. Thus, it indicates the effectiveness of the carboxymethylated derivative in suppressing the growth of the tumor cells.

It is recognized that a family of glucans consisting of (1−3)-β-D-glucan backbone with (1−6)-β-D-glucose from various

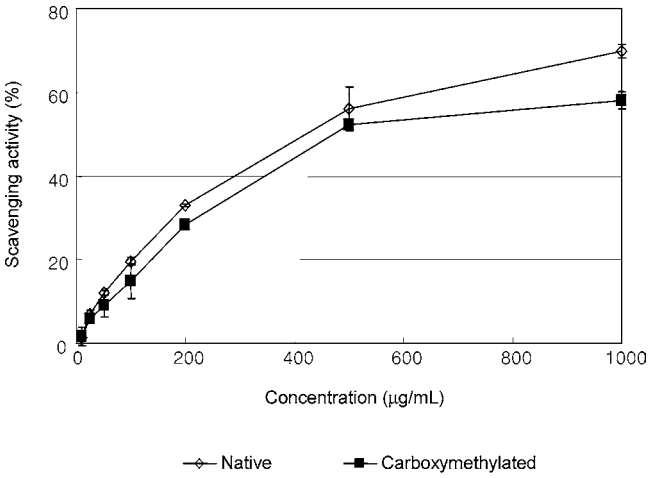

Figure 4. Effect of carboxymethylation of *P. linteus* polysaccharides on DPPH radical scavenging activity (the error bars indicate the standard deviation).

sources has cytotoxicity to tumor cells (*28*, *32*). Moreover, through chemical modifications, the incorporation of new functional groups with suitable degree of substitution can give polymers with improved cytotoxic activity. Specifically, the positive effect of sulfation and carboxymethylation on the cytotoxic activity of β-glucan could be attributed to increased water solubility and possible structural changes arising from macromolecular conformation (*11*, *12*, *24*). In addition, as mentioned in **Figure 2**, macrophage stimulation by the derivative might contribute to its increased cytotoxic activity against HT1080. However, because the carboxymethylated *P. linteus* polysaccharides showed little or no significant increase in the cytotoxic activity against two other tumor cells (MCF7 and SNU-C2A, data not shown), it appeared to have cytoxicity with specificity for particular tumor cells, which needs further investigation.

As a rapid and simple measure of antioxidant activity, the DPPH radical scavenging capacity has been widely used. The DPPH assay is based on the reduction of the stable radical DPPH to yellow-colored diphenylpicrylhydrazine in the presence of a hydrogen donor.

The effect of carboxymethylation on the DPPH radical scavenging activity of *P. linteus* polysaccharides was investigated as shown in **Figure 4**. Both samples were found to possess the DPPH radical scavenging activity, which increased as their concentrations increased to a certain extent and then appeared to reach a plateau. It was previously reported that *P. linteus* extract has a comparable antioxidant activity to vitamin C in scavenging the free radical DPPH (*33*). However, the derivative exhibited weaker activity to scavenge the DPPH radicals than the native one. The EC_{50} of the derivative was determined to be 470 μg/mL while that of the native *P. linteus* polysaccharides was 420 μg/mL. The carboxymethylation diminished the scavenging activities by 17% at a concentration of 1000 μg/mL. Therefore, the hydroxyl groups in the polysaccharides appeared to play an important role in maintaining its hydrogen-donating ability.

In summary, a chemical derivative of the polysaccharides extracted from *P. linteus* was prepared by carboxymethylation. The results showed that the carboxymethylated derivative exhibited the improvement of solubility, NO synthase, and in vitro cytoxic activities against HT1080.

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