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Acetylation and carboxymethylation of the polysaccharide from *Ganoderma atrum* and their antioxidant and immunomodulating activities

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ABSTRACT

A water-soluble polysaccharide extracted from *Ganoderma atrum* was chemically modified to obtain its acetyled and carboxymethylated derivatives. The results of chemical analysis, Fourier-transform infrared and ^{13}C nuclear magnetic resonance spectroscopy showed that these modifications were successful, although the molecular weight of these derivatives decreased due to slight degradation during the reaction. The antioxidant and immunomodulating activities of these derivatives were then investigated to determine the structure–bioactivity relationship. Results showed that the acetyled derivative with appropriate degree of substitution and lower molecular weight exhibited stronger antioxidant abilities on scavenging DPPH radical, and inhibitory effects in β-carotene–linoleic acid systems compared with the native polysaccharide. In addition, it also enhanced the macrophage phagocytosis capacity and tumor necrosis factor-α secretion, whereas the carboxymethylated derivative was shown to be slightly less effective. These results indicated that the type of substitution group and their degree of substitution play a decisive role in the bioactivities of the derivatives.

1. Introduction

Reactive oxygen species (ROS) are generated by normal metabolic processes or from exogenous pollutants. They are fundamental to many biochemical processes, such as the regulation of signal transduction and gene expression and activation of receptors. They also play a vital role in phagocytosis (Papas, 1999). On the other hand, accumulation of excessive ROS could result in oxidative stress and damage to DNA, proteins and other macromolecules, which are believed to attribute to various diseases, including asthma, rheumatoid arthritis, cardiovascular diseases, impairment of immune function and cancer (Valko et al., 2007). Thus, improved antioxidant status may have an immunostimulatory effect (Mau, Chao, & Wu, 2001). Antioxidant supplements or foods containing high concentrations of antioxidants may help reduce oxidative damage. Identification of new antioxidants remains a highly hot topic for researchers.

A number of natural polysaccharides have been recently demonstrated to play an important role as free radical scavengers in the prevention of oxidative damage in living organisms (Ananthi et al., 2010; Tseng, Yang, & Mau, 2008; Wang et al., 2009). Especially the mushroom-derived polysaccharides, such as polysaccharides from lentinan, schizophyllan, and krestin, have been accepted as immunoceuticals in Japan, Korea and China. Generally speaking, the antioxidant activity of polysaccharide depends on several structural parameters, such as degree of substitution (DS), degree of branching, molecular weight (MW), monosaccharide compositions and functional groups (Bohn & Be Miller, 1995). Therefore, increasing attention was paid to molecular modification and structure–activity relationship of the polysaccharides. Recently, many reports have demonstrated that some chemically modified polysaccharides exhibited improved biological activities than their corresponding natural polysaccharides (Liu, Luo, Ye, & Zeng, 2012; Ma, Chen, Zhang, Zhang, & Fu, 2012). Hence, chemical modifications of polysaccharides may provide an opportunity to obtain new agents with possible therapeutic uses.

Ganoderma atrum, a nutritious fungus belonging to the polyporaceae family of Basidiomycota, has been used as a functional food and preventive medicine for promoting health and longevity in Asian countries for more than 2000 years, and now has also become a popular dietary supplement in Western countries (Gao et al., 2005). Polysaccharides are the major bioactive constituents of *G. atrum*, contributing to the health-promoting effects and immunomodulating activity (Li et al., 2011). We have previously reported the preparation, structural characterization and biological

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activity of polysaccharide (PSG-1) from *G. atrum* (Chen, Xie, Nie, Li, & Wang, 2008; Zhang et al., 2011). PSG-1 is a branched heteropolysaccharide–protein complex, and the sugar moiety is mainly $-(1 \rightarrow 3)$, $-(1 \rightarrow 6)$, and $-(1 \rightarrow 4)$-linked glucan containing other sugar units such as galactose and mannose. *G. atrum* was found to contain a high level of crude polysaccharide (PSG which mainly consisted of PSG-1) with potential antioxidant and immunomodulating activities both *in vitro* and *in vivo* (Li et al., 2010, 2011). However, the structure–antioxidant activity relationship for this polysaccharide has not been elucidated.

For further research and utilization of polysaccharides from *G. atrum*, we report here the preparation, structural characterization, and some bioactivities *in vitro* of different derivatives of PSG. Firstly, different derivatives from PSG were prepared by means of acetylation and carboxylmethylation. Then, the structures of these derivatives were characterized by chemical analysis, Fourier-transform infrared (FT-IR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy. Finally, their antioxidant and immunomodulating activities *in vitro* were evaluated and compared, and the relationship between the chemical structure and antioxidant, immunomodulating activities of PSG was discussed.

2. Materials and methods

2.1. Materials

The fruiting bodies of *G. aturm* were collected from their original cultivation places located in Ganzhou, Jiangxi Province, China. The samples were identified by Dr. Zhi-hong Fu (Jiangxi University of Traditional Chinese Medicine). All of them were dried after collection to preserve and were then sliced and ground into fine powder in a mill before extraction.

1,1-Diphenyl-2-picrylhydrazyl (DPPH), linoleic acid, β-carotene, phenazine methosulfate (PMS), Coomassie Brilliant Blue G-250, ferrous chloride, ferrozine, ascorbic acid and butylated hydroxyl toluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dextrans of different MWs were purchased from Pharmacia Co., Ltd. Cell culture products were obtained from Life Technologies (Paisley, Scotland). Anti-tumor necrosis factor (TNF)-α antibody and rabbit anti-goat Ig-G/HRP conjugated-antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and solvents were of analytical reagent grade and were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of PSG and its derivatives

2.2.1. Preparation of PSG

The crude PSG was obtained according to our earlier study (Chen et al., 2008). Briefly, the air-dried fruiting bodies of *G. atrum* (1 kg) were ground and extracted with 20 l of hot water for 2 h. The solid residues were extracted with hot water twice. All filtrates were combined and concentrated to a final volume of 10 l and filtered. The filtrate was precipitated with ethanol and further deproteinized by Sevag reagent which was a $CHCl_3$/n-butanol (v/v = 5:1) mixture. The resulting aqueous fraction was extensively dialyzed against double-distilled water for 3 days and precipitated again by adding four fold volume of ethanol. The precipitate was collected by centrifugation and then washed with anhydrous EtOH. The fraction named PSG was obtained by dissolving in water and vacuum lyophilization. PSG was subjected to sulfation and acetylation modification for further research.

2.2.2. Preparation of acetylated derivative

The acetylation of PSG was carried out according to the method of Ma et al. (2012). To obtain acetylated derivatives with variable DS, three different amounts of acetylation reagent (acetic anhydride) were added, respectively during the derivation procedures. Briefly, PSG (300 mg) was dissolved in 10 ml distilled water. The mixture was stirred with a magnet stirrer until an homogeneous solution was obtained. The pH was adjusted to 9.0 with 0.5 M NaOH. The mixture was stirred at 30 °C for 4 h. During this period, the required amount of acetic anhydride was added, respectively at 40-min intervals. While simultaneously, 0.5 M NaOH was added to the mixture with continuous stirring to maintain the pH at 8.0–8.4. After reaction, the solution was neutralized with 5 M HCl to terminate the reaction, and dialyzed against distilled water with a 14,000 Da MW cut off membrane for 72 h. The aqueous solution was precipitated with ethanol. The resulting precipitate was dissolved in distilled water and freeze dried. According to the method described above, three kinds of acetylated derivatives were obtained and named as Ac-PSG-1, Ac-PSG-2 and Ac-PSG-3, respectively, which were correspondingly derived with 1, 3 and 5 ml acetic anhydride.

The acetyl group and degree of substitution (DS) of Ac-PSG were determined according to the method described by Das, Singh, Singh, and Riar (2010). DS is defined as the average number of sites per glucose unit that possess a substituent group, which was calculated as follows:

$$DS = 1.62 \times Ac/(43 - 0.42 \times Ac)$$

where Ac = % acetyl group (expressed as percentage on dry basis).

2.2.3. Preparation of carboxylmethylated derivative

The carboxymethylation of PSG was achieved by mixing 300 mg PSG with 12.5 ml isopropanol, and the mixture vigorously stirred for 15 min at room temperature. Then 5 ml of 20% NaOH was added drop wise. After being stirred at room temperature for 3 h, the carboxylmethylation agent (a mixture of 1.37 g or 2.63 g chloroacetic acid, 5 ml of 20% NaOH and 12.5 ml isopropanol) was added under stirring. The reaction was continued at 60 °C for 4 h. After the solution was cooled to room temperature, the pH value of the solution was adjusted to 7 with 0.5 M HCl. The product was dialyzed against flowing water for 12 h and then deionized water for 48 h. The non-dialyzable phase was concentrated and precipitated with 95% (v/v) ethanol at 4 °C for 12 h, then washed sequentially with ethanol, acetone and ether. The obtained sample was freeze-dried to get the carboxymethylated derivative and coded as CM-PSG-1 and CM-PSG-2, respectively, which were correspondingly derived with 1.37 and 2.63 g chloroacetic acid. The DS was determined using the neutralization titration method (De Paula, Heatley, & Budd, 1998).

2.3. Characterization of Ac-PSG and CM-PSG

The carbohydrate content of the derivatives was determined by a phenol–sulfuric acid method using D-galactose as a standard (Chen et al., 2008). The MWs of the samples was measured on Waters HPLC system equipped with a Waters Ultrahydrogel-500 column (7.8 × 300 mm) and a Waters 410 differential refractometer as described previously (Chen et al., 2008). Standard dextrans T-2000, T-500, T-150, T-100, T-70, T-40, T-10 and glucose were passed through the column. The calibration curves were obtained by plotting the elution volumes against the logarithm of their respective molecular weights. The elution volume of the tested polysaccharide was substituted into the equation of the calibration curve, and the molecular weight estimated.

FT-IR spectra were recorded on a Thermo Scientific Nicolet 5700 infrared spectrophotometer. The dried sample was ground with potassium bromide powder and pressed into a pellet for spectrometric measurement in the frequency range of 4000–400 cm^{-1}. ^{13}C NMR spectra were recorded at 500 MHz using a BruckerDRX-400