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Preparation, chain conformation and anti-tumor activities of water-soluble phosphated (1 → 3)-α-D-glucan from *Poria cocos* mycelia

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ABSTRACT

A water-insoluble (1 → 3)-α-D-glucan from *Poria cocos* mycelia was fractionated, followed by phosphorylation with H_3PO_4 in LiCl/Me_2SO containing urea to synthesize water-soluble phosphated derivatives. Their structures and chain conformations were investigated by FTIR, ^{31}P NMR, SEC-LLS and viscometry. The Mark–Houwink equation for the phosphated derivative in 0.15 M aqueous NaCl at 30 °C was established to be $[\eta] = 2.87 \times 10^{-3} M_w^{0.86\pm0.02}$. On the basis of conformational parameters calculated from wormlike cylinder model, the phosphated derivative existed as a semi-stiff chain in aqueous solution. Compared with unphosphated glucan, water-solubility and chain stiffness of the phosphated derivative increased, as a result of the introduction of phosphate group on main chain. All the phosphated derivatives exhibited significantly stronger anti-tumor activities than that of the unphosphated one, suggesting the effects of solubility and expanded chain conformation on improvement of the anti-tumor activity could not be negligible.

1. Introduction

Poria cocos is one of the most significant Chinese herbs, and polysaccharides from this fungus have attracted considerably attention in the fields of biochemistry and pharmacology because of their biological activities (Chen, Tang, Chen, Wang, & Li, 2010; Chihara, Hamuro, Maeda, Arai, & Fukuoka, 1970; Kanayama, Adachi, & Togami, 1983; Ke, Lin, Chen, Ji, & Shu, 2010; Lee et al., 2004; Lu, Cheng, Lin, & Chang, 2010; Wang, Yu, & Mao, 2009). In our previous work, water-soluble and water-insoluble polysaccharides were isolated from *P. cocos* mycelia produced in a pilot-scale fermenter. Significant anti-tumor activities were detected in water-soluble polysaccharides, but water-insoluble polysaccharide was the dominant product (90%) in the *P. cocos* mycelial extract and it exhibited hardly bioactivities (Huang, Jin, Zhang, Cheung, & Kennedy, 2007). The water-insoluble polysaccharide was confirmed to be (1 → 3)-α-D-glucan (Huang & Zhang, 2005), which belongs to the class of drugs known as biological response modifiers (BRMs). In this case, a major barrier to the utilization of (1 → 3)-α-D-glucan as BRMs is lack of solubility in aqueous media. If (1 → 3)-α-D-glucan is to become clinically applicable, it has to be converted into biologically effective, water-soluble form that can be safely administered via the systemic route.

It has been reported that phosphated polysaccharides exhibited anti-inflammatory, anti-tumor or anti-viral activities, especially immunomodulatory (Chen, Zhang, & Tian, 2002; Lyuksutova et al., 2005; Williams et al., 2004). Specifically, the phosphorylated *Achyranthes bidentata* polysaccharide with high degree of substitution was obtained when phosphorus oxychloride was used as phosphorylating agent, and it possessed anti-tumor activities against Sarcoma 180 and Lewis lung cancer in mice (Chen et al., 2002). Williams et al. have successfully prepared water-soluble glucan phosphate which was derived from water-insoluble (1 → 3)-β-D-glucan in *Saccharomyces cerevisiae* (Williams et al., 1991). Furthermore, they have extensively studied its immunobiological properties as well as mechanism, and focused efforts to understanding the chemical characteristics of β-D-glucan phosphate in order to associate structural determinant with biological activities. It is noted that the β-D-glucan phosphate can bind to immune cells by interacting with membrane receptors and stimulate various aspects of innate immunity (Lowman, Ensley, & Williams, 1998; Müller et al., 1995; Müller et al., 1996; Müller et al., 2000; Rice et al., 2002). The level of the polysaccharides bioactivities is closely related to their chemical composition, molecular weight, chain conformation, and water-solubility (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Young & Jacobs, 1998; Zjawiony, 2004). After phosphorylation, the water-solubility of polysaccharide will enhance, and molecular weight and chain conformation will be influenced by the presence of the charged phosphate groups. A basic understanding of both the primary and secondary structures (chemical composition, molecular weight and chain conforma-

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tion) for the phosphated polysaccharide is essential for successfully interpretation of their bioactivities.

In the present work, the objective was to converting water-insoluble (1 → 3)-α-D-glucan into water-soluble form, phosphated derivative, by phosphorylation. The dilution solution behavior, chain conformation and anti-tumor activities of the phosphated derivative compared with the unphosphated one were investigated and discussed. This work will contribute meaningful information to associate secondary structure (including molecular weight and chain conformation) with bioactivities of the biomacromolecules.

2. Materials and methods

2.1. Isolation and fractionation of Pi-PCM

A water-insoluble sample was isolated from *P. cocos* mycelia by extracting with 0.5 M NaOH/0.01 M $NaBH_4$ aqueous solution, followed by immediately precipitating with 1 M AcOH. The resultant precipitate was washed four times with distilled water and EtOH, respectively, and then lyophilized (Christ alpha 1-2, Osterode am Harz, Germany) to obtain white sample, coded as Pi-PCM (yield: 18.3%).

Pi-PCM was fractionated by the non-solvent addition method as follows. A certain amount of Pi-PCM was dissolved in 0.25 M LiCl/Me_2SO to obtain a clear solution with concentration of 0.8%. A mixture of acetone and 0.25 M LiCl/Me_2SO (4:1, v/v) as precipitant was slowly added to the Pi-PCM solution at 25 °C until the solution became slightly milk-white. Then the turbid solution was heated to 50 °C with stirring. After being brought to 25 °C and standing for 12 h, the turbid solution was centrifuged (7000 rpm, 15 min) at 25 °C to separate the concentrated phase as first fraction. The supernatant was subjected to next fractionation. In this way, the Pi-PCM sample was divided into fourteen fractions. The fractions were reprecipitated from 0.25 M LiCl/Me_2SO solutions by the addition of 80% aqueous acetone, and then washed with anhydrous acetone four times and vacuum-dried for seven days to obtain white powder. Nine of the fractions, coded as F1–F9, were selected to be used for investigating molecular weight and dilution solution behaviors.

2.2. Preparation of phosphated derivative

As shown in Fig. 1, nine of the fractions F1 to F9 were phosphorylated individually by the improved method for Williams et al. (1991). The fraction (200 mg) was dissolved in 0.25 M LiCl/Me_2SO containing 3.6 g urea. About 1.5 mL of H_3PO_4 (85%) was added dropwise slowly to the above solution at ambient temperature. Then the solution was heated to 100 °C, and the reaction was carried out for 6 h with stirring. A crystalline precipitate (presumed ammonium phosphate) formed at 1–2 h of reaction. Following heating, the reaction mixture was cooled to ambient temperature and diluted in distilled-water to form a yellow clear solution. Finally, the resulting phosphated derivative was dialyzed against distilled water for seven days to remove endotoxin (including Me_2SO, H_3PO_4 and salt), and then lyophilized to obtain white flocculent samples labeled as P1–P9. The phosphated derivatives obtained by lyophilization were vacuum-dried for three days to eliminate a little amount of water and then stored in a desiccator containing P_2O_5.

2.3. Structure analysis

IR spectra were recorded using the KBr-disk method with a Nicolet Fourier transform infrared (FTIR) spectrometer (Spectrum One, Thermo Nicolet Co., Madison, WI, USA) in the range 400–4000 cm^{-1}. ^{31}P NMR spectrum was recorded on an Inova-600 MHz NMR spectrometer (Varian Inc., Palo alto, CA, USA) in order to confirm the presence of phosphate group as substitution. The

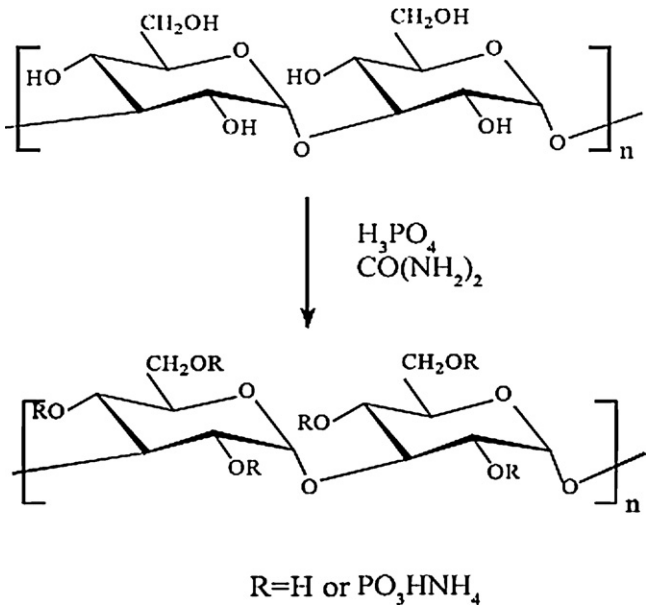

Fig. 1. Phosphorylation of (1 → 3)-α-D-glucan from *Poria cocos* mycelia with phosphoric acid.

phosphated derivative was dissolved in 99.96% D_2O and its concentration was adjusted to 5 wt%. H_3PO_4 (85%) was used as external reference substance (δ_{31P} = 0 ppm). The phosphorus content of the phosphated derivative was determined on an inductively coupled plasma–atom emission spectrograph (IRIS Intrepid 11 XSP, Thermo Electron Co., USA).

2.4. Viscometry

The instinct viscosities ([η]) of the phosphated derivatives (P1–P9) in 0.15 M NaCl aqueous solution were measured at 30 ± 0.1 °C by using a Ubbelohde capillary viscometer. The effluent time for the solvent was always beyond 120 s, so the kinetic energy correction was negligible. Huggins and Kraemer equations were used to estimate [η] by extrapolating to infinite dilution formulated as follows:

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c \quad (1)$$

$$\frac{\ln \eta_r}{c} = [\eta] - \beta[\eta]^2 c \quad (2)$$

where k' and β are constants for a given polymer under given conditions in a given solvent; η_{sp}/c, the reduced specific viscosity; and $(\ln \eta_r)/c$, inherent viscosity.

2.5. SEC-LLS measurements

Size exclusion chromatography with on-line multi-angle laser light scattering (SEC-LLS) measurements were carried out on a DAWN® DSP laser photometer (DAWN® DSP, Wyatt Technology Co., Santa Barbara, CA, USA), combined with TSK-GEL PWXL G5000 column (7.8 mm × 300 mm) and equipped with a P100 pump (Thermo Separation Products, San Jose, CA, USA) for the phosphated derivatives in 0.15 M aqueous NaCl at 30 °C. An interferometric refractometer (Optilab, DAWN® DSP, Wyatt Technology Co., Santa Barbara, CA, USA) was simultaneously connected. The calibration of the laser photometer was done with ultra pure toluene and the normalization was done with pullulan standard (Shodex Standard P-10, $M_w = 1.18' \times 10^4$, $M_w/M_n = 1.10$, Showa Denko, Japan) at the concentration of 5–7 mg mL^{-1}. The calibration of the interferometric refractometer was made with NaCl aqueous solution. The