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Chain conformation and anti-tumor activities of phosphorylated (1→3)-β-D-glucan from *Poria cocos*

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ABSTRACT

(1→3)-β-D-Glucan isolated from *Poria cocos* was phosphorylated to obtain a series of phosphorylated derivatives. Their structures, weight-average molecular weights (M_w), and chain conformation were studied by ^{13}C NMR, ^{31}P NMR, static laser light scattering and viscometry. The experimental results revealed that the phosphorylated glucan existed as relatively extended flexible chain in 0.15 M NaCl aqueous solution, and exhibited relatively strong inhibition against S-180 tumor cell *in vitro* and *in vivo*. *In vivo*, the fractions with relatively high molecular weight at low dosage exhibited stronger anti-tumor activities. The results revealed that the molecular weights and molecular conformation could influence the anti-tumor activities. The molecular weight ranging from 2.6×10^4 to 26.8×10^4 and the extended chain conformation were beneficial to enhance the anti-tumor activity, as a result of the increasing of the interaction between polysaccharide and immune system.

1. Introduction

The studies on the fungal polysaccharides have attracted attention in the fields of pharmacology because of their non-toxicity and anti-tumor effects (Jeong, Yang, Jeong, Kim, & Song, 2008; Lavia, Friesemb, Gereshc, Hadarb, & Schwartz, 2006; Ye, Wang, Zhou, Liu, & Zeng, 2008; Zhang, Cui, Cheung, & Wang, 2007). Polysaccharides are regarded as biological response modifier (BRM) because they are harmless and help the body to adopt to environmental and biological stress (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Yan et al., 1999). Many anti-tumor drugs such as 5-fluorouracil can kill tumor cells as well as normal cells and are therefore toxic. Some polysaccharides can only destroy the tumor cells without affecting the normal ones, and their anti-tumor activities mostly resulted from their immunomodulating effects (Hsieh et al., 2008; Togola et al., 2008). Normally some polysaccharides can stimulate granulocytes, monocytes, macrophages and NK-cells and trigger the secretion of IFN-g, IL-6, IL-8 and IL-12 from macrophages, neutrophils and NK-cells (Ladanyi, Timar, & Lapis, 1993). It is noted that polysaccharide derivatives have shown stronger anti-tumor activities than the unmodified polysaccharide (Bao, Nie, Chen, Liu, & Tao, 2007; Tao, Zhang, & Cheung, 2006). Particularly, phosphorylated derivatives of polysaccharides have exhibited attenuates cardiac dysfunction, anti-inflammatory, antioxidant activity and antimicrobial immunity (Sherwood et al., 2001; Williams et al., 2004; Yuan et al., 2005). However, phosphorylated polysaccharides as anti-tumor agent have scarcely been published.

Polysaccharides with different structures have been found to exist as various chain conformations in solution (Huang & Zhang, 2005; Patel et al., 2008; Tao & Zhang, 2006; Yang & Zhang, 2008; Zhang, Zhang, & Cheung, 2003). The molecular weight and chain conformation of the polysaccharides significantly affected their bioactivities (Falch, Espevik, Ryan, & Stokke, 2000; Kojima, Tabata, Itoh, & Yanaki, 1986; Wolfgang, Johannan, Josef, & Gerhard, 1992). (1→3)-β-D-Glucan in the triple helical state can suppress growth of S-180 tumor, but its random coil state was found to be ineffective (Yanaki et al., 1983). The anti-tumor activities of triple helical lentinan decreases significantly with the transition to a single chain. (Maeda, Watanabe, Chihara, & Rokutanda, 1988; Surenjav, Zhang, Xu, Zhang, & Zeng, 2006; Zhang, Li, Xu, & Zeng, 2005). Therefore, a basic understanding of the molecular weight, conformation of the phosphorylated polysaccharide in aqueous solution is essential for successful interpretation of the bioactivities mechanism of the polysaccharides.

Poria cocos is a kind of fungi and has been used as a traditional medicine (Chen et al., 2009). (1→3)-β-D-Glucan extracted from *P. cocos* sclerotium is its main component, but it is water-insoluble and shows no anti-tumor activity (Ding, Zhang, & Zeng, 1998). Usually, chemical modification of water-insoluble polysaccharide can improve their water solubility. The molecular weight, molecular conformation, and anti-tumor activity of the phosphorylated (1→3)-β-D-glucan of *P. cocos* have never been reported. In the

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present work, we attempted to synthesize phosphorylated (1→3)-β-D-glucan derivatives and to study the molecular weight, molecular chain conformation of the phosphorylated polysaccharide derivative as well as its anti-tumor activities *in vitro* and *in vivo*. It may contribute some information to anti-tumor mechanism of the polysaccharides.

2. Experimental

2.1. Preparation of sample

The sclerotium of *P. cocos* was cultivated in Luotian (Hubei, China). The fresh sclerotium was powdered, and the sun dried sclerotium powder was defatted sequentially by using Soxhlet extractor with ethyl acetate for 6 h and then with acetone for 6 h. The resultant residue was immersed in 0.15 M aqueous NaCl at 25 °C. The mixture was stirred for 24 h, centrifuged at $5.478 \times 10^3 g$ to obtain the residue. The residue was immersed in distilled water at 120 °C for 40 min, and then centrifuged. The resulting residue was extracted with 0.5 M NaOH aqueous solution for 4 h, centrifuged to get the supernatant fluid. The resulting supernatant fluid was neutralized with 50% acetic acid solution, centrifuged to obtain the precipitates, coded as PCS3-II. PCS3-II was then dissolved in dimethyl sulphoxide (Me_2SO), and was fractionated by following non-solvent addition method. A mixture of acetone and Me_2SO at the volume ratio of 4:1 was slowly added into PCS3-II solution at 25 °C until the solution turned slightly milky. The turbid mix was warmed up to 50 °C to become transparent again. After being brought to 25 °C and standing for 12 h, the turbid solution was centrifuged to separate into liquid and gel phase. The gel was removed and the supernatant was subjected to the next step of fractionation. In this way, PCS3-II was divided into 7 fractions. Each fraction was re-precipitated by acetone, then washed with anhydrous acetone three times, and finally vacuum-dried to yield white powder.

Williams's method was used to synthesize phosphorylated PCS3-II (Williams et al., 1991). 400 mg of PCS3-II fraction and 7.2 g urea were dissolved in 20 mL Me_2SO, and 3 mL H_3PO_4 was dropped into the above solution at 100 °C to react for 6 h. Subsequently the solution cooled down, and was dialysed thoroughly against distilled water to remove Me_2SO and urea. The dialysed solution was lyophilized to dry to yield phosphorylated derivative, coded as P-PCS3-II. The P-PCS3-II fractions were coded as P1-PCS3-II, P2-PCS3-II, P3-PCS3-II, P4-PCS3-II, P5-PCS3-II, P6-PCS3-II, and P7-PCS3-II.

2.2. Structure characterization

Phosphorus content in the derivative was determined on an ICP–AES spectrometer (IRIS Intrepid II XSP, Thermo Electron Corporation). ^{13}C NMR measurement was analyzed on a Mercury 600 NMR spectrometer (Varian Inc., USA) at 20 °C. P-PCS3-II was dissolved in D_2O to obtain polysaccharide solution with a concentration of 80 mg/mL. ^{31}P NMR measurement was analyzed on a DRX-400 NMR spectrometer (BRUKER, Germany) at 20 °C. P-PCS3-II was dissolved in D_2O to obtain polysaccharide solution with a concentration of 30 mg/mL. 85% H_3PO_4 was used as external standard.

2.3. Molecular weight measurements

Weight-average molecular weight (M_w) of the P- PCS3-II fractions were measured with a laser light scattering instrument equipped with a He–Ne laser (MALLS, $\lambda = 633$ nm; DAWN®DSP, Wyatt Technology Co., Santa Barbara, CA, USA) at multiple angles (θ) of 43°, 60°, 69°, 80°, 90°, 100°, 111°, 121°, and 132° at 25 °C. The basic light scattering equation is as follows:

$$\frac{Kc}{R_\theta} = \frac{1}{M_w}\left[1 + \frac{16\pi^2 n^2 < S^2 >_z}{3\lambda^2} \cdot \sin^2\left(\frac{\theta}{2}\right)\right] + 2A_2 c + \cdots \quad (1)$$

where K is an optical constant equal to $[4\pi^2 n^2 (dn/dc)^2]/(\lambda^4 N_A)$; c is the polymer concentration in g/mL; R_θ is the Rayleigh ratio; λ is the wavelength; n is the refractive index of the solvent; dn/dc is the refractive index increment; N_A is the Avogadro' number; A_2 is the second viral coefficient. The polysaccharide solution with desired concentrations was prepared, and optical clarification of the solution was achieved by being filtrated through a 0.2 μm pore size filter (PTFE, Puradisc 13-mm Syringe Filters, Whatman, England) into a scattering cell. ASTRA software (version 4.90.07) was utilized for data acquisition and analysis. The refractive index increment (dn/dc) was measured with a double-beam differential refractometer (Optilab, Wyatt Technology Co., Santa Barbara, CA, USA) at the wavelength of 633 nm at 25 °C. The dn/dc average value of the P1-PCS3-II-P7-PCS3-II fractions in in 0.15 M NaCl aqueous solution was 0.131 $cm^3\,g^{-1}$.

2.4. Intrinsic viscosity measurement

Intrinsic viscosities ($[\eta]$) of the P-PCS3-II fractions in 0.15 M NaCl aqueous solution were measured at 25 ± 0.1 °C using an Ubbelohde capillary viscometer. The kinetic energy correction was assumed to be negligible. Huggins and Kraemer equations were used to estimate the $[\eta]$ value by extrapolating to an infinite dilution formulated as follows:

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

$$(\ln \eta_r)/c = [\eta] - \beta[\eta]^2 c \quad (3)$$

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

2.5. In vitro anti-tumor activity against sarcoma 180 tumor cell

Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method was used to measure the proliferation of adherent tumor cells. The Sarcoma 180 tumor cells were inoculated on a 96-well cultivation plate at a concentration of 1×10^4 cells/mL. Each cell was inoculated with 100 μL Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum solution containing the tumor cells and 20 μL samples (at concentrations of 0.005, 0.05, 0.5, 5 mg/mL in 0.15 M NaCl, respectively) under an atmosphere of 5% CO_2 at 37 °C for 24 h. The tumor cells were continuously inoculated for another 4 h after addition of 10 μL MTT (5 mg/mL). The supernatant was removed by centrifuging, and then 100 μL Me_2SO was added to terminate the reaction. The survival rate of the tumor cells was assayed by measuring the optical intensity by an auto enzyme-labeled meter (CliniBio 128, Australia) at 550 nm. The sample groups were compared with control group in the absence of the tested samples. All *in vitro* results were expressed as the inhibition ratio (Φ) of tumor cell proliferation as follows:

$$\Phi = [(OD_a - OD_b)]/OD_a] \times 100\% \quad (4)$$

where OD_a is the absorbance value of negative control group and OD_b is that of sample group, respectively. All assays were made in triplicate. 0.005 mg/mL 5-Fluorouracil (5-Fu) in 0.15 M NaCl aqueous solution was used as positive control. 0.15 M NaCl was used as negative control.