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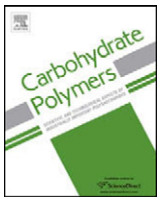

Preparation, chain conformation and anti-tumor activities of water-soluble phosphated (1 → 3)-α-D-glucan from *Poria cocos* mycelia

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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

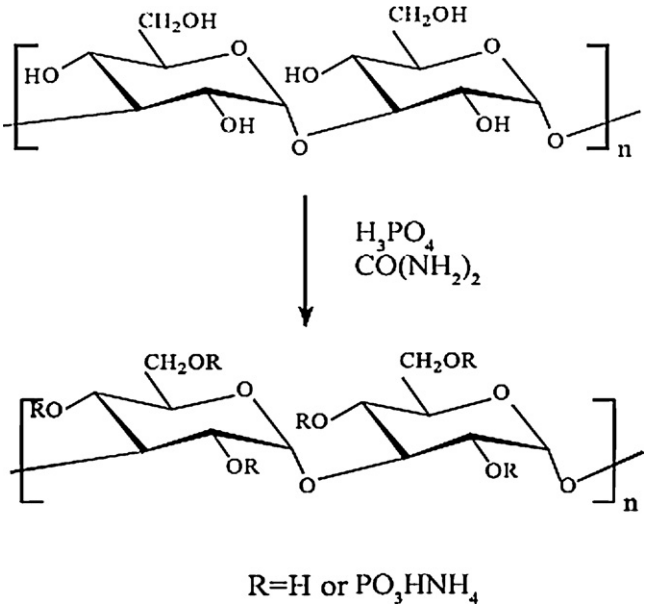

Fig. 1. Phosphorylation of (1 → 3)-α-D-glucan from *Poria cocos* mycelia with phosphoric acid.

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′ × 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

average value of refractive index increment (dn/dc) for the phosphated derivatives (P1–P9) in 0.15 M NaCl was determined to be 0.130 mL g^{-1} using the interferometric refractometer at 633 nm and 30 °C. As a carrier solution, 0.15 M NaCl aqueous solution was dust-free treated by passing through 0.2 (m pore size filter membrane (Jinteng Co., Tianjin, China) and degassed before being used. The injection volume was 200 μL with the concentration of 3 mg mL^{-1}, and the flow rate was 0.5 mL min^{-1}. Astra software (Version 4.90.07) was utilized for data acquisition and analysis.

2.6. In vitro anti-tumor activity assay

Sarcoma 180 (S-180) tumor cell provided by Tongji Medical College of HuaZhong University of Science and Technology (Wuhan, Hubei, China) was utilized *in vitro* anti-tumor test. Colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method was used for measuring the proliferation of S-180 cells. S-180 cells were inoculated on a 96-well cultivation plate at a concentration of 1×10^6 cells/mL, and grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) containing the samples at final concentrations of 0.005, 0.05 and 0.5 mg/mL under an atmosphere of 5% carbon dioxide at 37 °C for 24 h. After 10 μL MTT (5 mg/mL) added, the tumor cells were continuously incubated for another 4 h. Finally, the media was removed by centrifugation, and Me_2SO was added with slight shaking to dissolve the crystal formazan of reaction product between MTT and living cells. The survival rate of the tumor cells was assayed by measuring optical density (*OD*) on an auto enzyme-labeled meter (Bio-Tek EL×800, USA) at 540 nm. The tested groups were compared with the negative control group in the absence of the tested samples and the positive control group of 5-Fluorouracil. All of *in vitro* results were expressed as the inhibition ratio (Φ) of tumor cell proliferation as follows:

$$\Phi = \left[\frac{OD_c - OD_t}{OD_c}\right] \times 100\% \quad (3)$$

where OD_c and OD_t are the average value of optical density for the negative control and tested group, respectively. All samples were carried out in triplicate.

2.7. In vivo anti-tumor activity assay

The *in vivo* anti-tumor activity tests of the phosphated derivatives were carried out on S-180-bearing mouse model. S-180 tumor cells were subcutaneously inoculated (5×10^6 cells/mouse) into 8-week-old BALB/c female mice with body weight about 20 g. The phosphated derivatives were dissolved separately in 0.9% aqueous NaCl; the Pi-PCM sample was suspended in the same solvent as the phosphated derivatives. The samples solutions were injected intraperitoneally (i.p. 20 mg/kg) once daily for 8 days starting 24 h after tumor inoculation. The same volume of 0.9% aqueous NaCl was injected i.p. into the negative control mice and 5-Fluorouracil in 0.9% aqueous NaCl was injected i.p. into the positive control mice. The mice were killed after the tumors were allowed to grow for another 7 days, and the tumors were excised and weighed. The inhibition ratio (ξ) and enhancement ratio of body weight (f) were calculated as follows:

$$\xi = \left[\frac{W_c - W_t}{W_c}\right] \times 100\% \quad (4)$$

$$f = \left[\frac{W_a - W_b}{W_b}\right] \times 100\% \quad (5)$$

where W_c and W_t are the average tumor weight of the negative control and tested groups; W_b and W_a are the body weight of mice before and after the assay. Statistical evaluations in all experiments were performed by SPSS software. Significant difference between the control and tested group was defined as $p < 0.05$.

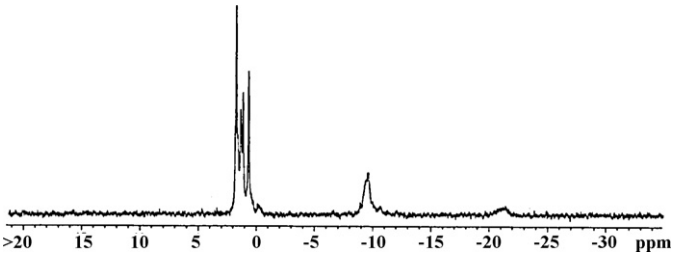

Fig. 2. ^{31}P NMR spectrum of the phosphated (1 → 3)-α-D-glucan in D_2O.

3. Results and discussion

3.1. Chemical structure

Compared with the FTIR spectrum of the native Pi-PCM (figure not shown), a new absorption peak appeared at 1230 cm^{-1} for the phosphated derivative assigned to H-bonded P=O stretching vibration, indicating that the phosphated reaction has actually occurred (Inoue, Kawamoto, Nakajima, Kohno, & Kadoya, 1983; Williams & Fleming, 1980). A C-O-P stretching signal was usually found at 1055–950 cm^{-1}, but it was overlapped by three strong characteristic absorption bands of glucopyranoside residue at 1100–1010 cm^{-1} (Granja et al., 2001a). As shown in Fig. 2, ^{31}P NMR spectrum of the phosphated derivative exhibited an intense and multi-signal peak for phosphorus resonance in the region of 0.4–1.3 ppm. The results confirmed that phosphate groups were bound to the molecular chain, and indicated that phosphate groups were located in several different magnetic environments ascribed to the substitution at different position such as C-6, C-2 or C-4 (Granja et al., 2001b; Lowman et al., 1998). In addition, the second small peak was observed at −9.8 ppm, suggesting that another type of phosphate group was present in the phosphated derivative. Similar findings when phosphorylating chitin fibers by the urea/H_3PO_4 reagent have been reported by Yokogawa et al. (1997), which was speculated that the small peak at −9.9 ppm was attributed to the ammonium hydrogen phosphate formed.

The degree of substitution (DS), designed the average number of phosphate group on each glucose residue, could be calculated from phosphorus content on the basis of the formula as follows (Sitohy, Labib, EI-Saadany, & Ramadan, 2000):

$$\text{DS of momoammonium esters} = \frac{162 \times P}{3100 - 9700 \times P} \quad (6)$$

where P = % phosphorus of the phosphated derivatives. The phosphorus content and DS of the phosphated samples are summarized in Table 1. With a decrease of molecular weight of the Pi-PCM fractions, DS of the corresponding phosphated derivatives increased under the same conditions. The results revealed that it was easy for the low M_w fractions to introduce phosphate groups on the backbone, as a result of allowing more reagent molecules to approach the reactive hydroxyl groups. Compared with the unphosphated and water-insoluble Pi-PCM fractions, the water solubility in range of 10–20 mg/mL for the phosphated derivatives was improved greatly, so the investigation for molecular weight and chain conformation in aqueous solution could become possible.

3.2. Molecular weight and intrinsic viscosity

Usually, 0.15 M aqueous NaCl (0.9% NaCl) as a medium is extensively used in various bioactivity assays. The investigating of M_w, viscosity and molecular conformation for the phosphated deriva-

Table 1
The experimental results of SEC-LLS, viscometry and ICP-AES for the phosphated derivatives (P1–P9) at 30 °C in 0.15 M NaCl aqueous solution.

Original fractions	$M_w \times 10^{-4}$ [a]	Phosphated derivatives	$M_w \times 10^{-4}$	M_w/M_n	$[\eta]$	P %	DS
F1	57.3	P1	15.6	2.33	81.7	0.96	0.052
F2	45.2	P2	9.29	2.12	53.6	1.00	0.054
F3	37.5	P3	7.87	1.94	47.4	1.43	0.078
F4	30.8	P4	5.67	1.97	35.0	1.40	0.077
F5	28.3	P5	3.50	2.15	24.2	1.95	0.109
F6	23.0	P6	3.45	1.44	–	2.01	0.112
F7	17.4	P7	3.14	1.93	19.5	2.78	0.159
F8	11.1	P8	2.98	1.40	–	2.81	0.161
F9	7.75	P9	1.96	1.38	14.2	2.79	0.160

–: not detected because of M_w close to that of P5 or P7.
[a] Data obtained in our previous work (Huang & Zhang, 2005).

tive in such medium is essential to clarify the correlation of the secondary structure to bioactivities for biomacromolecules. Thus, 0.15 M aqueous NaCl was used as solvent for the phosphated derivatives, and the viscosity measurements indicated that electrostatic repulsion had been eliminated. The SEC patterns of the phosphated derivatives in 0.15 M aqueous NaCl, analyzed by the laser light scattering detector (LLS detector 11 = 90°) and by the interferometric refractometer (AUX detector), are present in Fig. 3. Obviously, each phosphated derivative with different M_w was eluted at different elution volume in order from high to low M_w. Moreover, each sample exhibited a single peak detected by LLS, suggesting that there was no detective aggregation of the phosphated derivative in the aqueous solution. Slight peak following main peak only detected by AUX detector resulted from very-low M_w substance, which was attributed to a small quantity of salt remaining in phosphated product. The values of M_w, M_w/M_n and $[\eta]$ for the phosphated derivatives are summarized in Table 1, compared with the unphosphated corresponding fractions. From Table 1, the molecular weight distributions of the derivatives were relatively narrow with the polydispersity index (M_w/M_n) from 1.4 to 2.3. Hence, the phosphated derivatives were suitable for investigation of their solution properties. It was noted that the M_w values of the phosphated derivatives were lower than that of the unphosphated one as a result of the degradation of macromalecules in the phosphorylation process.

3.3. Mark–Houwink equation

The effect of DS for the phosphated polysaccharides on the $[\eta]$–M_w relationship could be neglected because the value of DS was small and the electrostatic interaction was screened as a result of normal viscosity behavior in 0.15 M aqueous NaCl similar to neutral polymer. Fig. 4 shows M_w dependence of $[\eta]$ for the phosphated derivatives (P1–P9) in 0.15 M NaCl aqueous solution at 30 °C. The Mark–Houwink equation for the phosphated derivative in the M_w range from $1.96' \times 10^4$ to $15.6' \times 10^4$ was established as follows:

$$[\eta] = 2.87 \times 10^{-3} M_w^{0.86\pm0.02} \times (\text{mL g}^{-1}, \text{ in 0.15 M NaCl aqueous solution}) \quad (7)$$

The exponent (α) is related to the shape and backbone structure of the macromolecular and nature of the solvent. For a flexible polymer in good solvent, the α value is usually in the range from 0.5 to 0.8. When the macromolecule chain is more expanded, or presents semi-stiff, the α value is above 0.8 (Shukla et al., 1991). The α value of 0.75 for the Pi-PCM sample in 0.25 M LiCl/Me_2SO was located in upper limit of flexible chain, as reported in our paper (Huang & Zhang, 2005). Interestingly, the phosphated derivative possessed a higher α value of 0.86 than the unphosphated Pi-PCM, suggesting that its chain was more expanded, and existed as a semi-stiff chain in aqueous media.

3.4. Conformation parameters

On the basis of data of M_w and $[\eta]$, a wormlike cylinder model could be used for conformational characterization of the phosphated derivative. Bohdanecky has proposed that the Yamakawa–Fujii–Yoshizaki (Y–F–Y) theory for $[\eta]$ of an unper-

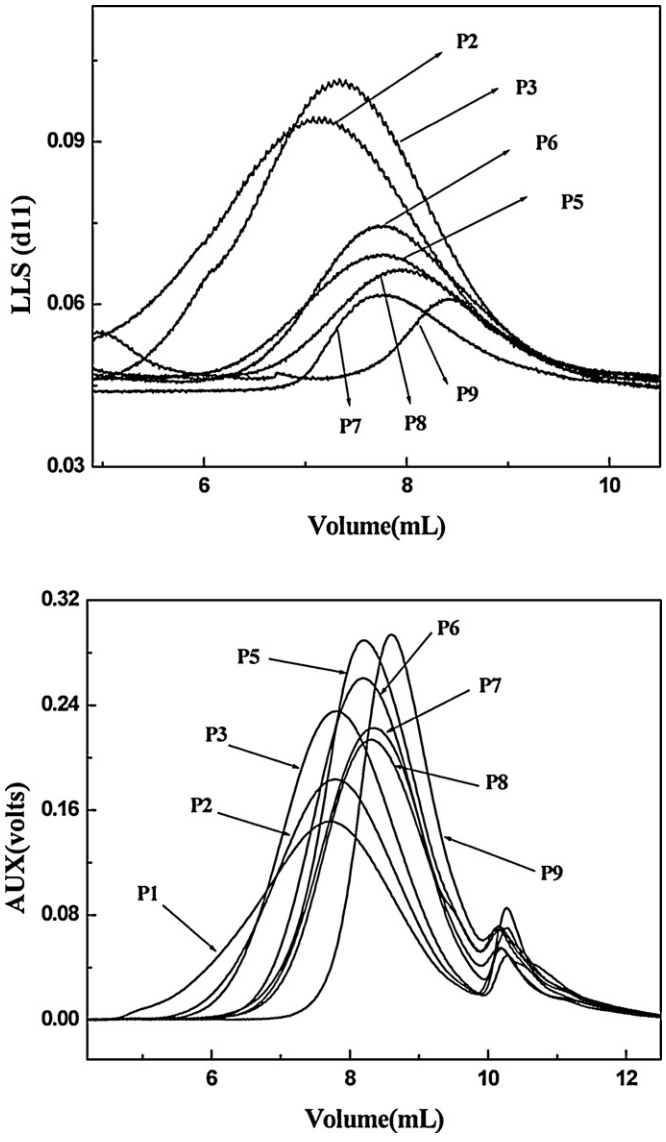

Fig. 3. SEC of the phosphated derivatives in 0.15 M NaCl aqueous solution at 30 °C determined by LLS detector (d11) (top), and by AUX detector (bottom).

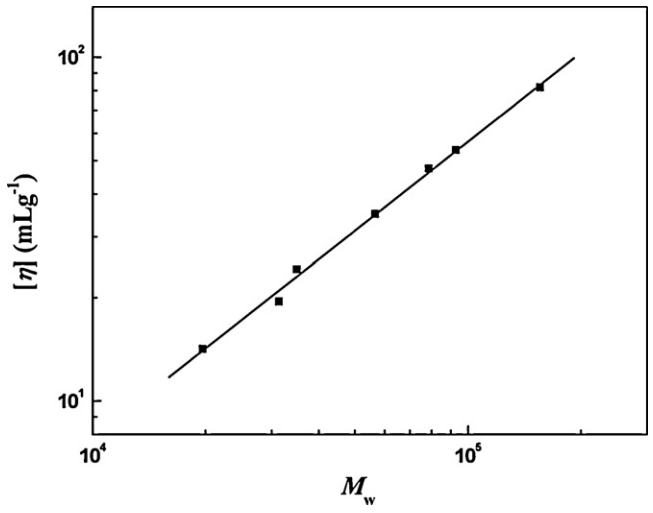

Fig. 4. M_w dependence of $[\eta]$ for the phosphated derivatives in 0.15 M NaCl aqueous solution at 30 °C.

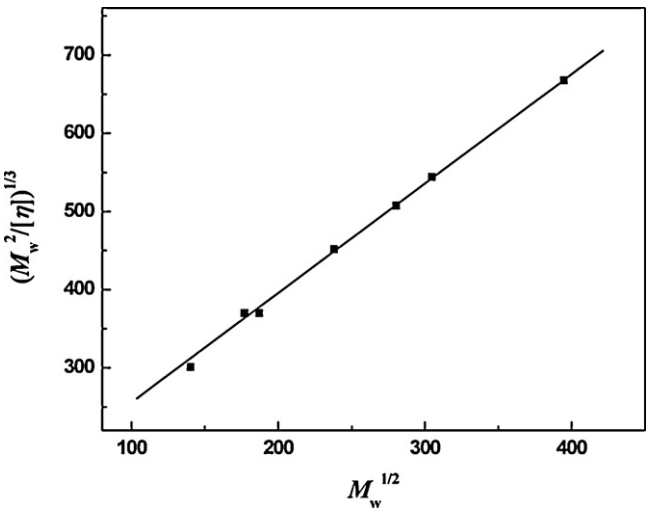

Fig. 5. Plot of $(M_w^2/[\eta])^{1/3}$ against $M_w^{1/2}$ for the phosphated derivatives in 0.15 M NaCl aqueous solution at 30 °C.

turbed wormlike cylinder could be represented approximately as follows (Bohdanecky, 1983; Yamakawa & Fujii, 1974; Yamakawa & Yoshizaki, 1980):

$$\left(\frac{M^2}{[\eta]}\right)^{1/3} = A_\eta + B_\eta M^{1/2} \tag{8}$$

$$A_\eta = \phi_{0,\infty}^{-1/3} A_0 M_L \,(\mathrm{g}^{1/3}\,\mathrm{cm}^{-1}) \tag{9}$$

$$B_\eta = \phi_{0,\infty}^{-1/3} B_0 \left(\frac{2q}{ML}\right)^{-1/2} \quad \text{or}$$

$$= \phi_{0,\infty}^{-1/3} B_0 \left(\frac{\langle R_0^2\rangle}{M}\right)_\infty^{-1/2} (\mathrm{g}^{1/3}\,\mathrm{cm}^{-1}) \tag{10}$$

where q, M_L and $\langle R_0^2\rangle$ are the persistence length, the molar mass per unit contour length and mean-square end to end distance, respectively. A_0 and B_0 are tabulated in Bohdanecky's paper (Bohdanecky, 1983), and $\phi_{0,\infty}$ the Flory viscosity factor for unperturbed random coils, is 2.86×10^{23} mol^{-1}. On the base of M_w and $[\eta]$ data, $(M_w^2/[\eta])^{1/3}$ was plotted against $M_w^{1/2}$ for the phosphated derivatives as shown in Fig. 5. Substituting of the intercept and slope of this plot into Eqs. (8)–(10) yielded 780 ± 40 nm^{-1} for M_L and 6.0 ± 1 nm for q of the phosphated derivative. Compared with the data of the unphosphated Pi-PCM ($M_L = 760 \pm 50$ nm^{-1}) Huang & Zhang, 2005, the slightly increasing value of M_L for the phosphated derivative suggested that the phosphated polysaccharide included side groups or short branches, which was consistent with presence of phosphate group substitution. (Xu, Zhang, Nakamura, & Norisuye, 2002)

The characteristic ratio (C_∞) represents the degree of the chain extension as a result of restrictions on both internal rotation and bond angle. C_∞ is defined as follows (Nakanishi, Norisuye, Teramoto, & Kitamura, 1993)

$$C_\infty = \frac{M_0}{\lambda M_L l^2} \tag{11}$$

where M_0 is the average molar mass of a glucose residue in repeat unit, λ^{-1} is the Kuhn's segment length ($\lambda^{-1} = 2q$), and l is the virtual bond length equal to the distance between two successive glycosidic oxygens O(3) and O(3′). Considering the effect of the substituted group on the glucose residue, M_0 was calculated to be 173 as $[162 + 97 \times \mathrm{DS_{aver}}]$, where $\mathrm{DS_{aver}}$ is the average value of DS for P1–P9. l may be 0.425 nm for glucopyranose of the phosphated derivative (Nakanishi et al., 1993), together with $\lambda^{-1} = 12.0 \pm 2$ nm, $M_L = 780 \pm 40$ nm^{-1}, C_∞ of the phosphated derivative in 0.15 M NaCl was calculated to be 15.1 ± 1. The C_∞ value was significantly larger than that of amylose, (1 → 4)-α-D-glucan in Me_2SO ($C_\mu = 5.0$) (Nakanishi et al., 1993), and that of curdlan, (1 → 3)-β-D-glucan in 0.3 M NaOH aqueous sulution ($C_\mu = 10.5$) (Nakata, Kawaguchi, Kodama, & Konno, 1998). The results indicated that the phosphated derivative molecules with main chain structure of (1 → 3)-α-D-glucan were more extended than those of the (1 → 4)-α-D-glucan and the (1 → 3)-β-D-glucan, which was in good agreement with the conclusion from Burton and Brant (1983). On the basis of DS (0.05–0.16), it could be concluded that only a phosphate group substitution was present on the average every 10 glucose subunit. Such low introduction of phosphate groups on main chain could not result in an evidently increasing of steric hindrance, as well as chain stiffness. Hence, C_∞ (15.1 ± 1) of the phosphated derivative in 0.15 M NaCl was slightly higher than that of the unphosphated one ($C_\infty = 14.9 \pm 0.2$) (Huang & Zhang, 2005), suggesting the phosphated (1 → 3)-α-D-glucan existed as a more expanded chain.

In view of the conformation parameters (q, M_L and C_∞), the phosphated (1 → 3)-α-D-glucan in aqueous solution existed as a more expanded, semi-stiff chain. Its water-solubility and chain stiffness may be favorable for some bioactivities (Williams et al., 1991). Therefore, its anti-tumor activities and the correlation of molecular weight and chain conformation to anti-tumor activities were continuously investigated.

3.5. In vitro results

The c dependence of *in vitro* inhibition ratio to Sarcoma 180 tumor cell (S-180) proliferation by the unphosphated Pi-PCM and phosphated derivatives are shown in Fig. 6. Significant anti-tumor activity was not found in the unphosphated Pi-PCM, whereas all the phosphated derivatives exhibited considerably stronger inhibition ratio against S-180 cell growth at tested concentration levels. Furthermore, obvious dose-response relationship of the phosphated derivatives on suppressing S-180 cells proliferation was observed. From the semi-log graph, the IC50 values of the various phosphated derivatives were evaluated to be 0.01–0.2 mg/mL, and IC 50 of P1–P4 with higher M_w were lower than that of P7 and P9 having lower M_w, indicating that relatively high molecular weight was favorable to suppression of tumor cells proliferation. Interestingly, the phosphated derivatives (P1–P5) in the M_w range from

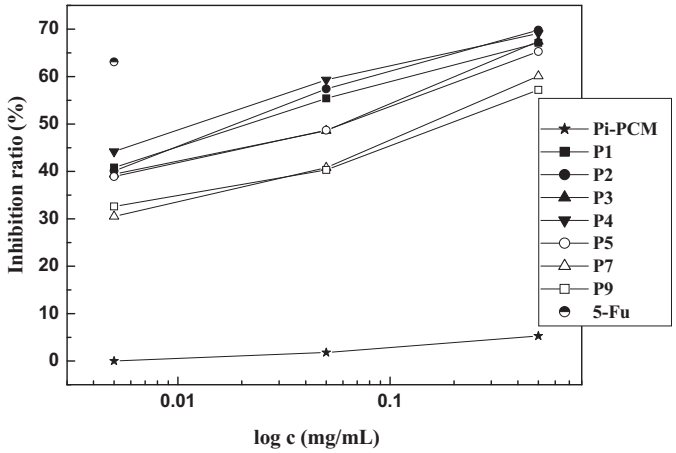

Fig. 6. *C* dependence of *in vitro* inhibition ratio against Sarcoma 180 tumor cells growth by the Pi-PCM and phosphated derivatives.

$15.6' \times 10^4$ to $3.50' \times 10^4$ at concentration of 0.5 mg/mL exhibited considerably strong inhibition ratio of 67.1%, 69.8%, 67.4%, 69.1% and 65.3% against S-180 cell growth, respectively, which exceed 5-Fu (63.1% at 0.005 mg/mL). It is well known that 5-Fu is toxic owing to killing cancer cells as well as normal cells, whereas polysaccharides from natural products are relatively safer. It has been reported in our previous work that the sulfated glucan exhibited stronger *in vivo* and *in vitro* anti-tumor activities against S-180 cell than the unsulfated one (Huang, Zhang, Cheung, & Tian, 2006). In our finding, the phosphated glucan had similar anti-tumor effects to the sulfated glucan, suggesting that the introduction of charged and hydrophilic groups, such as phosphated and sulfated groups on the backbone resulted in the enhancement of anti-tumor activities.

3.6. In vivo results

The results of the *in vivo* anti-tumor activities of the phosphated derivatives and the unphosphated Pi-PCM for comparison against Sarcoma 180 tumor cells (S-180) are summarized in Table 2. No obvious anti-tumor activity was detected in the unphosphated Pi-PCM, indicating that it was ineffective in suppressing the growth of S-180 cells. However, all the phosphated derivatives with M_w range from $1.96' \times 10^4$ to $15.6' \times 10^4$ showed more potent anti-tumor activities than the unphosphated Pi-PCM. Moreover, the enhancement ratios of body weight for all the phosphated derivatives were significantly higher than that for 5- Fu (26.1%) and even for the sulfated derivatives (20–40%) (Huang et al., 2006), indicating that the phosphated (1 → 3)-α-D-glucan had higher safety and showed promising utilization as anti-tumor drugs. It was noted that the phosphated derivatives (P1–P4) having comparatively moderate M_w ($M_w > 5.5' \times 10^4$) showed pronounced inhibition ratio of more than 50% ($p < 0.01$) compared with the lower (M_w = 3.5, 3.14, $1.96' \times 10^4$), indicating the influence of M_w on bioactivities of the phosphated derivatives. It has been reported and approved that relatively moderate molecular weight in range of $5' \times 10^4$ to $50' \times 10^4$ was favorable to enhance the anti-tumor activities of polysaccharide and either too low or too high molecular weight would decrease the anti-tumor activities (Bohn & BeMiller, 1995; Wasser, 2002; Zhang, Cheung, & Zhang, 2001; Zhang, Chen, & Xu, 2005).

Table 2
The results of the *in vivo* anti-tumor activities of the phosphated derivatives and the unphosphated Pi-PCM for comparison against Sarcoma 180 tumor cells.

Samples	Dose (mg/kg × days)	Inhibition ratio (ξ%)	Enhancement ratio of body weight (*f*%)
Pi-PCM	20 × 8	9.41	40.9
P1	20 × 8	62.5[a]	42.6
P2	20 × 8	55.7[a]	40.5
P3	20 × 8	54.1[a]	39.7
P4	20 × 8	50.8[a]	34.6
P5	20 × 8	34.2[b]	42.2
P7	20 × 8	30.6[b]	40.7
P9	20 × 8	24.3	49.0
Control	20 × 8		53.6
5-Fu	20 × 8	52.4[a]	26.1

[a] Compared with the control group $p < 0.01$.
[b] Compared with the control group $p < 0.05$.

4. Conclusion

Water-soluble α-glucan derivatives having deferent molecular weight were satisfactorily synthesized from the corresponding fractions of water-insoluble (1 → 3)-α-D-glucan by phosphorylation, H_3PO_4 used as phosphorylating reagent and LiCl/Me_2SO containing urea as solvent. The Mark–Houwink equation for the phosphated derivative in 0.15 M NaCl aqueous solution at 30 °C was established to be $[\eta] = 2.87 \times 10^{-3} M_w^{0.86\pm0.02}$ (mL g^{-1}). On the basis of conformation parameters (M_L, q and C_∞), the phosphated derivative of (1 → 3)-α-D-glucan existed as a semi-stiff chain in 0.15 M NaCl aqueous solution. Compared with the unphosphated α-glucan, water-solubility and chain stiffness of the phosphated derivative increased, as a result of the introduction of phosphate group at hydroxyl group on the backbone. The phosphated derivatives exhibited significantly *in vivo* and *in vitro* anti-tumor activities against Sarcoma 180 tumor cell compared with the unphosphated one, suggesting that the effect of solubility and expanded chain on improvement of the anti-tumor activities could not be negligible. In addition, the relatively moderate molecular weight ($5' \times 10^4$ to $50' \times 10^4$) was favorable to enhancement of the anti-tumor activities for polysaccharide.

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