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Carbohydrate Polymers 70 (2007) 324–333
Carbohydrate Polymers
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Structure, molecular size and antitumor activities of polysaccharides from *Poria cocos* mycelia produced in fermenter

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Received 21 March 2006; received in revised form 21 April 2007; accepted 24 April 2007
Available online 6 May 2007

Abstract

Mycelia of *Poria cocos* were obtained from a pilot-scale 50-L fermenter by submerged cultivation biotechnology. Six polysaccharide fractions were extracted sequentially from the *P. cocos* mycelia with 0.9% NaCl (Pi-PCM1), hot water (Pi-PCM2), 0.5 M NaOH (Pi-PCM3-I and -II), and 88% formic acid (Pi-PCM4-I and -II). The supernatant of the culture media was spray-dried to obtain the extracellular polysaccharide (Pi-PCM0) produced by the mycelia. The results from Fourier transform infrared spectroscopy, gas chromatography, and ^{13}C NMR revealed that the water-soluble fractions including Pi-PCM0, Pi-PCM1, and Pi-PCM2 were heteropolysaccharides mainly containing glucose, galactose, and mannose, whereas Pi-PCM3-I and Pi-PCM4-I had (1 → 3)-α-D-glucan characteristics. The weight-average molecular mass (M_w) and intrinsic viscosity ([η]) of the polysaccharide were measured by laser light scattering (LLS) and viscometry, respectively. Results indicated that Pi-PCM1 and Pi-PCM2 existed as a compact random coil in aqueous solution, close to the global shape. Interestingly, the three water-soluble polysaccharides (Pi-PCM0 to Pi-PCM2) all exhibited antitumor activities *in vivo* (Sarcoma 180 solid tumor implanted in BALB/c mice), and *in vitro* (HL-60 tumor cell).

Keywords: *Poria cocos* mycelia; Pilot-scale submerged cultivation; Polysaccharide structure; Antitumor activity

1. Introduction

During the past 30 years, a plethora of polysaccharides have been isolated from plants, fungi, yeasts, mushrooms, and sea organisms by extraction (Berovič et al., 2003; Zhao, Kan, Li, & Chen, 2005). The high molecular weight polysaccharides derived from the cell walls, often called biological response modifiers (BRM) or immunopotentiators, can prevent carcinogenesis, show direct antitumor activity against various allogeneic and syngeneic tumors, as well as preventing tumor metastasis (Chen & Chang, 2004; Zjawiony, 2004). Therefore, to discover and evaluate polysaccharides with antitumor and immunostimulating properties has emerged as one of important research fields in chemistry, and biology (Zhang & Huang, 2005).

Poria cocos (Fu-Ling in China) has been widely used as a Chinese traditional herbal medicine for centuries (Chen & Chang, 2004). It has been reported that polysaccharides extracted from *P. cocos* have antitumor and immunomodulatory activities (Chen & Chang, 2004; Chihara, Hamuro, Chang, Maeda, Arai, & Fukuoka, 1970; Kanayama, Adachi, & Togami, 1983; Lee et al., 2004). The level of the polysaccharides' bioactivities is closely related to their chemical composition, molecular weight, branching, chain conformation, and water-solubility (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Young & Jacobs, 1998; Zjawiony, 2004). Therefore, a basic understanding of both

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doi:10.1016/j.carbpol.2007.04.015

the primary and secondary structures for the polysaccharides is essential for successfully interpretation of their bioactivities. Recently, *P. cocos* mycelia have been produced in a pilot-scale 50-L fermenter by means of submerged cultivation biotechnology (Zhang, Jin, Chen, & Cheung, 2004). However, polysaccharides isolated from *P. cocos* mycelia produced in fermenter have been little reported. There is also very little information on the structures, molecular parameters, and bioactivities of the polysaccharides. Furthermore, there is the question as to whether high temperatures, e.g. the inlet temperature of 250 °C of spray-driers have an influence on bioactivities of extracellular polysaccharide. To address these information shortcomings, six polysaccharides were extracted from *P. cocos* mycelia obtained in 50-L fermenter, and the supernatant of the culture media was spray-dried to obtain the extracellular polysaccharide. The chemical composition, molecular mass (M_w), chain conformation, and intrinsic viscosity ([η]) of these polysaccharides were determined by Fourier transform infrared spectroscopy (FTIR), gas chromatography (GC), ^{13}C NMR, laser light scattering (LLS), and viscometry. Moreover, *in vitro* and *in vivo* antitumor activities for three water-soluble polysaccharides were evaluated.

2. Materials and methods

2.1. Submerged cultivation of P. cocos mycelia

Submerged cultivation of *P. cocos* mycelia was carried out in a pilot-scale fermenter as previously described (Zhang et al., 2004). A strain of *P. cocos* coded as No. P0 was obtained from wild *P. cocos* sclerotium in Luotian (Hubei, China). Previous work confirmed that the *P. cocos* polysaccharides produced from the wild strain No. P0 in medium containing corn steep liquor exhibited the highest antitumor activities (Jin, Zhang, Zhang, Chen, & Cheung, 2003a). Therefore, the wild strain was grown and inoculated into 40-L liquid medium containing corn steep liquor, D-glucose, yeast extract, and minerals, with a pH value adjusted to 5.0–6.0. The submerged cultivation was performed in 50-L fermenter with an air flow volume of 2–3 m^3/h with stirring rate of 150 rpm at 28 °C for 7 days. The resulting mycelia were separated by filtration, washed with distilled water five times, and then vacuum-dried (code Pi-PCM). In addition, the supernatant from the culture medium was spray-dried (PSD52, APV ANHYDRO-AS, Denmark) with inlet and outlet temperatures of 250 and 70 °C, respectively, to yield the crude extracellular polysaccharide (Pi-PCM0-C).

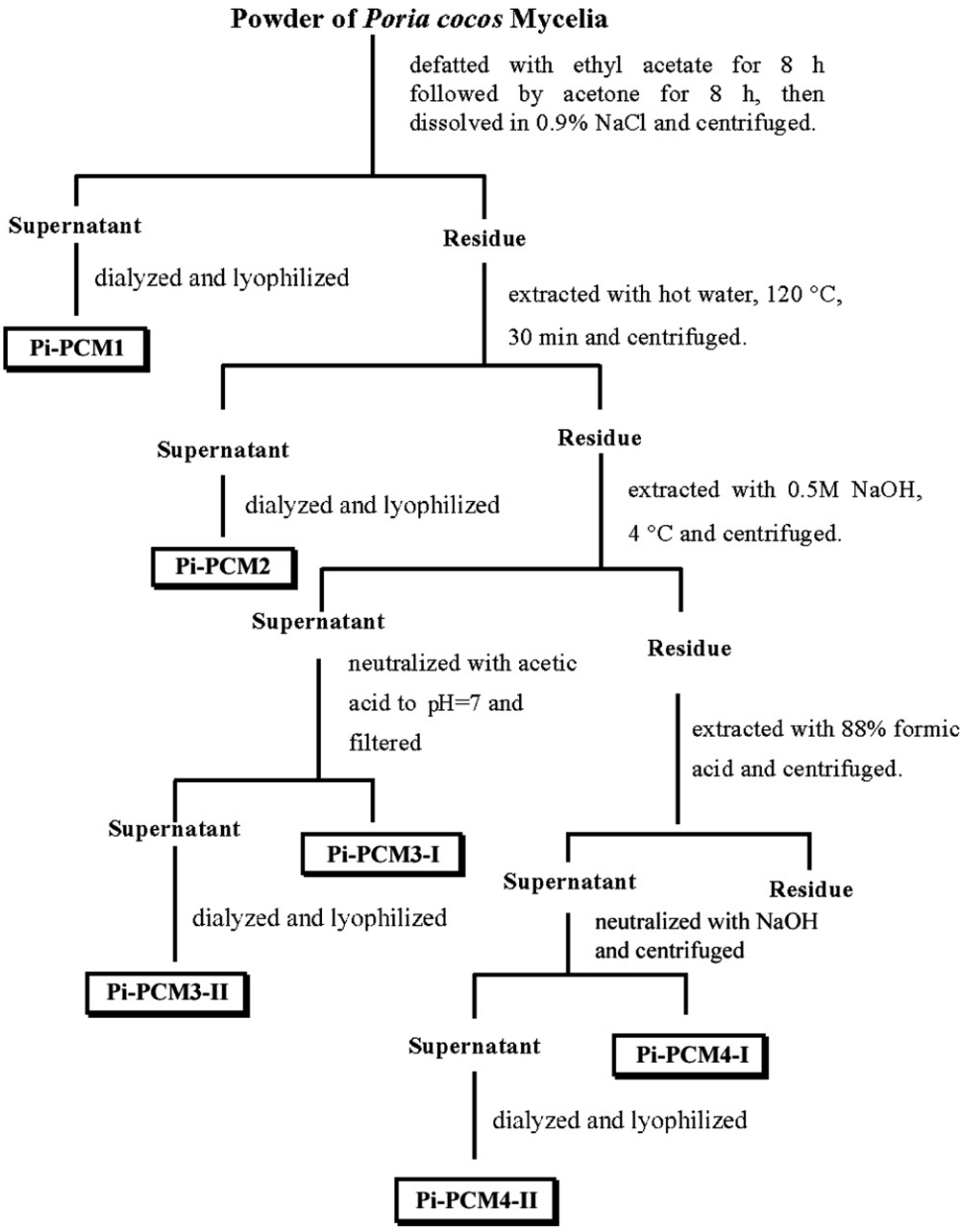
2.2. Isolation and purification of polysaccharides

Poria cocos mycelia were defatted by Soxhlet extraction with ethyl acetate for 8 h and then acetone for 8 h. The resulting residue was powdered by milling and then immersed in 0.9% aqueous NaCl overnight before being centrifuged to give the supernatant. For decolorisation 30% H_2O_2 (200 mL) was added slowly to the supernatant (2000 mL). To remove free protein Sevag's method was used (addition of 5:1 v/v chloroform and *n*-butanol to the supernatant) and stirred vigorously to precipitate free protein at the boundary between the aqueous and organic phases. It was then dialyzed using regenerated cellulose tube (M_w cut-off 8000, Union Carbide, NJ, USA) against tap water for 5 days and distilled water for 3 days, concentrated by rotary evaporation at reduced pressure below 42 °C and lyophilized (Christ alpha 1–2, Osterode am Harz, Germany) to obtain the first polysaccharide fraction coded as Pi-PCM1. Subsequent extractions of the *P. cocos* mycelial polysaccharides by hot water, 0.5 M NaOH, and 88% formic acid were carried out as shown in Scheme 1. Consequently, six polysaccharides, coded as Pi-PCM1, Pi-PCM2, Pi-PCM3-I, Pi-PCM3-II, Pi-PCM4-I, and Pi-PCM4-II, were isolated from the *P. cocos* mycelia. In addition, the crude extracellular polysaccharide (Pi-PCM0-C) was redissolved in distilled water and purified as described above to yield pure extracellular polysaccharide, coded as Pi-PCM0.

2.3. Analysis of chemical composition

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}. High-resolution ^{13}C NMR spectra were recorded on an Inova-600 MHz NMR spectrometer (Varian Inc., Palo alto, CA, USA). The polymer concentration was adjusted to 5 wt% in all experiments. D_2O (99.96%) was used as solvent for Pi-PCM0, Pi-PCM1, and Pi-PCM2 with acetone as an internal reference ($\delta c = 31.45$). 0.25 M LiCl/dimethylsulfoxide-d_6 (Me_2SO-d_6) was used as solvent for Pi-PCM3 and Pi-PCM4.

The carbohydrate content of the samples was determined spectrophotometrically by a phenol-sulfuric acid method (Dubois, 1956). GC analysis of the alditol acetate derivatives of the sugars was carried out by the method of Englyst, Quigley, and Hudson (1994). Samples were hydrolyzed with 12 M sulfuric acid for 1 h below 35 °C followed by dilution to 2 M and heated at 100 °C for 2 h. After neutralization with ammonia, sodium borohydride solution was added in order to reduce aldoses to alditols. Finally, the alditols were acetylated with acetic anhydride. Gas chromatography of the alditol acetate derivatives of the sugars was performed on a HP6890 gas chromatograph (Agilent, USA) fitted with a flame ionization detector and a capillary column (30 m × 0.75 mm i.d. Supelco SP-2330). The GC condition was: injector temperature, 280 °C; column temperature, 220 °C; detector temperature, 280 °C; carrier gas, nitrogen; and flow rate, 8 cm^3/min. The uronic acid content of the samples was measured by spectrophotometry according to the colorimetric method (Englyst et al., 1994). Two weight percent sodium chloride, 3 wt%



Scheme 1. Sequential extraction system of polysaccharides from *Poria cocos* mycelia.

boric acid solution (0.3 mL) was poured to the hydrolysates mentioned in GC procedure. After concentrated sulfuric acid (5 mL) had been added, the solutions were heated at 70 °C for 40 min, and then cooled to room temperature. Dimethylphenol (0.2 mL) as chromogenic reagent was added and vortex-mixed immediately. After 15 min, the absorbance of the samples was measured at 450 and 400 nm. The reading at 400 nm was subtracted from that at 450 nm to eliminate the interference from hexoses.

2.4. Viscometry

The viscosities of Pi-PCM0, Pi-PCM1, and Pi-PCM2 in 0.2 M aqueous NaCl, and Pi-PCM3 and Pi-PCM4 in 0.25 M LiCl/Me_2SO were measured at 25 °C by using an Ubbelohde capillary viscometer. The flow time, for two solvent systems, 0.2 M sodium chloride, and 0.25 M LiCl in dimethyl sulfoxide were always beyond 120 s, the kinetic energy correction was negligible. Huggins and Kraemer equations were used to estimate intrinsic viscosity $[\eta]$ by extrapolation to infinite dilution as follows (Huggins, 1942; Kraemer, 1938):

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

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

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

2.5. Laser light scattering

The light-scattering intensities of the polysaccharide solutions were determined with a multi-angle laser light scattering (LLS) instrument equipped with a He–Ne laser ($\lambda = 633$ nm; DAWN® DSP, Wyatt Technology Co., Santa Barbara, CA, USA) at the angles from 26° to 142° at 25 °C. The calibration of the laser photometer was done with ultra pure toluene and the normalization was done with pullulan standards (Shodex Standard P-10, M_w=1.18 × 10^4, $M_w/M_n = 1.10$, Showa Denko, Japan) at the concentrations of 2–3 mg mL^{-1}. The optical clarification of the polysaccharide solution and solvent was achieved by filtration through Millipore filters (0.45 μm for 0.25 M LiCl/Me_2SO; 0.2 μm for 0.2 M aqueous NaCl) into the scattering cell K5. The values of dn/dc were determined using an interferometric refractometer (Optilab, DAWN® DSP, Wyatt Technology Co., Santa Barbara, CA, USA) at 633 nm and 25 °C. The dn/dc values of samples in 0.2 M NaCl and in 0.25 M LiCl/Me_2SO were 0.142 and 0.055 mL g^{-1}, respectively. Astra software (Version 4.90.04, Wyatt Technology Co., Santa Barbara, CA, USA) was utilized for data acquisition and analysis.

2.6. In vitro proliferation and cytotoxicity assays

HL-60 leukemia cells (1 × 10^6 cells/mL) were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum under an atmosphere of 5% carbon dioxide at 37 °C for 72 h containing the samples at concentrations of 50, 100, and 200 μg/mL in phosphate-buffered saline (8.812 g NaCl, 0.201 g KCl, 0.204 g KH_2PO_4, and 1.150 g Na_2HPO_4 in 1 L ultra-pure water, pH 7.4). The survival rate of the HL-60 cells was assayed by counting living cells that excluded the Trypan blue dye using a hemacytometer (Jin et al., 2003a; Zhang, Zhang, Cheung, & Ooi, 2004).

Human MCF-7 cells and Vero cells (1 × 10^6 cells/mL) were incubated separately with the samples at concentrations of 50, 100, and 200 μg/mL and allowed to grow under the same condition as the HL-60 cells mentioned above. The number of living MCF-7 cells and Vero cells at end of the 72-h incubation period was determined in triplicate by a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide as described by Mosmann (1983). In the above two assays, 5-fluorouracil as a positive control and the tested samples were compared with negative control in the absence of Pi-PCM0, Pi-PCM1, and Pi-PCM2. All *in vitro* results were expressed as the inhibition ratio (Φ) of tumor cell proliferation as follows:

$$\Phi = [(A - B)/A] \times 100\% \quad (3)$$

where A and B are the average number of viable tumor cells for the control and samples, respectively.

2.7. In vivo antitumor test

Sarcoma 180 cells (1 × 10^5 cells/mouse) were subcutaneously inoculated into 8-week-old BALB/c male mice with body weight about 20 g. 5-Fluorouracil as a positive control and the tested samples (Pi-PCM0, Pi-PCM1, and Pi-PCM2) dissolved in phosphate-buffered saline (pH 7.4) were injected intraperitoneally (i.p. 20 mg/kg) into the mice once daily for 10 days starting 24 h after tumor inoculation. The same volume of phosphate-buffered saline (pH 7.4) was injected i.p. into the negative control mice. The tumor was allowed to grow on the mice for another 7 days before it was removed from the mice and weighed. The antitumor activities of the tested samples were expressed as the inhibition ratio (ξ) calculated as follows:

$$\xi = [(W_c - W_t)/W_c] \times 100\% \quad (4)$$

where W_c and W_t are the average tumor weight of the control and tested groups, respectively.

2.8. Statistical analyses

Student's t-test was used to evaluate the differences between the control and tested group. Significant difference between two groups was defined as $p < 0.05$.

3. Results and discussion

3.1. Chemical composition

In the IR spectra of the samples Pi-PCM0 to Pi-PCM4-II (Fig. 1) all exhibited the characteristic absorption of polysaccharides at 1650, 1400, and 1250 cm^{-1}. The IR spectrum of the extracellular polysaccharide Pi-PCM0 had absorption peak at 810 cm^{-1}, which was characteristic of mannan (Mathlouthi & Koenig, 1986). The absorption peaks at 810 or 870 cm^{-1} in the IR spectra of Pi-PCM1, Pi-PCM2, Pi-PCM3-II, and Pi-PCM4-II were assigned to mannan and galactan (Mathlouthi & Koenig, 1986). The polysaccharide Pi-PCM4-II had an absorption peak at 890 cm^{-1}, implying the existence of β-D-glucan. In contrast, the obvious characteristic peak of α-D-glucan appeared at 850 and 920 cm^{-1} in Pi-PCM3-I as well as Pi-PCM4-I (Dighton, Mascarenhas, & Arbuckle-Keil, 2001; Sandula, Kogan, Kacurakova, & Machova, 1999).

Monosaccharide compositions obtained by GC is shown in Table 1, together with uronic acid and total carbohydrate content. The results indicated that carbohydrate was the dominant component in these extracts, of which Pi-PCM3-I and Pi-PCM4-I were pure carbohydrate. The glucose content rapidly increased to become the predominant monosaccharide during the sequential extractions. The Pi-PCM0, Pi-PCM1, and Pi-PCM2 polysaccharides were determined to be heteropolysaccharides containing mannose, galactose, and glucose as major sugar and traces of other monosaccharides. Mannose

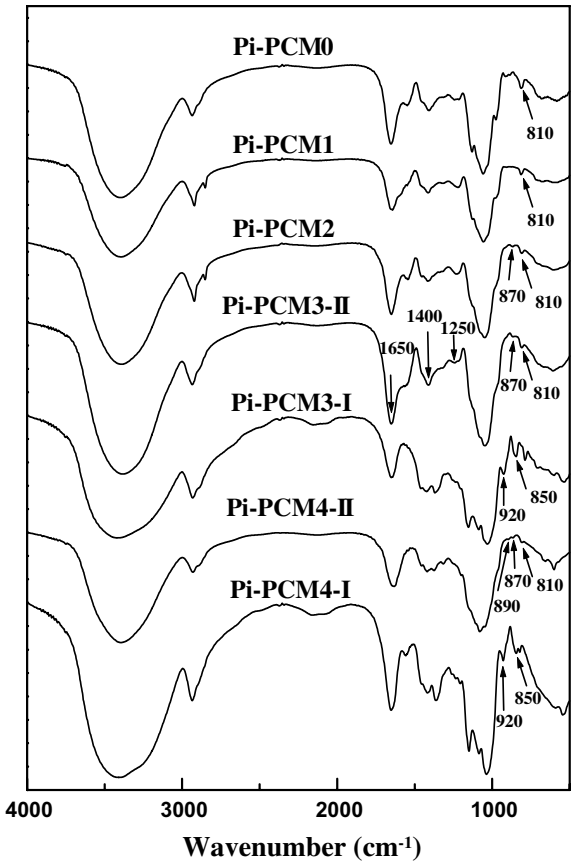


Fig. 1. The IR spectra of the samples Pi-PCM0 to Pi-PCM4-II.

(70.6%) and galactose (45.6%) was present at the highest amount in Pi-PCM0 and Pi-PCM4-II, respectively. Notably, Pi-PCM3-I and Pi-PCM4-I contained only glucose. In addition, there was an extremely low level of uronic acid in all polysaccharides, which was in good agreement with the results from NMR.

In the ^{13}C NMR spectra of the five polysaccharides (Pi-PCM2, Pi-PCM3-I and -II, Pi-PCM4-I and -II) (Fig. 2), Pi-PCM3-I and Pi-PCM4-I exhibited only one anomeric carbon peak in the anomeric region, indicating that these polysaccharides were composed of only one sugar type. The signals around 99.7 (99.6), 70.9 (71.0), 82.4 (82.2), 69.4 (69.6), 71.9 (72.0), and 60.1 (60.3) ppm, assigned to C-1, C-2, C-3, C-4, C-5, and C-6, were identical with those of an (1 → 3)-α-D-glucan (Jin, Zhang, Chen, Chen, & Cheung, 2003b; Chen, Zhou, Zhang, Nakamura, & Norisuye, 1998), confirming that Pi-PCM3-I and Pi-PCM4-I were linear (1 → 3)-α-D-glucans. The ^{13}C NMR spectrum of Pi-PCM3-II showed not only the characteristic peaks of (1 → 3)-α-D-glucan at 99.8 (C-1), 71.0 (C-2), 82.4 (C-3), 69.5 (C-4), 72.0 (C-5), and 60.4 (C-6), but also the typical signals of β-D-galactofuranoside residues at 106.9 (C-1), 106.6 (C-1′), 80.1 (C-2), 76.2 (C-3), 82.4 (C-4), 73.6 (C-5), and 69.5 (C-6) (Gorin, 1981; Nagaoka et al., 1996). These results suggested that (1 → 3)-α-D-glucan and β-D-galactan co-existed in the Pi-PCM3-II polysaccharide in accord with the results of GC. The strong signals in the spectrum of Pi-PCM4-II at 102.9, 72.8, 86.0, 68.4, 76.3, and 60.8 ppm should be attributed to (1 → 3)-β-D-glucan characteristic absorption at C-1, C-2, C-3, C-4, C-5, and C-6 (Misaki, Kakuta, Sasaki, Tanaka, & Miyaji, 1981; Saito et al., 1987). The other anomeric signal in the spectrum of Pi-PCM4-II at 106.6 ppm accompanied with 81.7 and 82.1 ppm could be assigned to C-1, C-2, and C-4 of β-D-galactofuranoside residues. Thus Pi-PCM4-II possibly contained (1 → 3)-β-D-glucopyranoside and β-D-galactofuranoside residues, confirming the results from GC (Table 1). The ^{13}C NMR spectrum of Pi-PCM2 contained three C-1 resonances at δ_c 107.3, 102.6, and 99.7 in anomeric region. The lowest field C-1 signal at 107.3, together with 81.7 (C-2), 76.8 (C-3), 83.2 (C-4), 73.6 (C-5), and 70.2 (C-6), corresponded to β-linked D-galactofuran. The chemical shift in the highest field of C-1 peak at 99.7 with 70.8 (C-2), 82.0 (C-3), 72.0 (C-5), and 60.7 (C-6) were assigned to (1 → 3)-α-D-glucan. Additionally, the characteristic signals of mannan appeared at 102.6 (C-1), 70.2 (C-2, 3), 67.3 (C-4), 73.6 (C-5), and 61.5 (C-6) (Vinogradov, Petersen, & Duus, 2000; Xu et al., 2004). The ^{13}C NMR data accompanied with GC and IR results indicated that Pi-PCM2 was a heteropolysaccharide composed of β-D-galactofuranan, (1 → 3)-α-D-glucan and mannan.

3.2. Molecular mass and intrinsic viscosity

By performing a batch mode on the LLS detector, weight-average molecular mass (M_w) and z-average radius of gyration ($\langle s^2 \rangle_z^{1/2}$) of polymer can be obtained directly by

Table 1
Monosaccharide composition, uronic acid, and total carbohydrate content in all samples from *Poria cocos* mycelia

Sample	Monosaccharide content in polysaccharide (%)						Uronic acid (%)	Total carbohydrate content (%)
	Fuc	Ara	Xyl	Man	Gal	Glc		
Pi-PCM0	–	2.5	1.5	70.6	18.5	7.0	0.5	82.6
Pi-PCM1	10.9	1.0	2.8	23.6	36.5	25.2	0.3	86.5
Pi-PCM2	1.9	–	–	29.6	38.9	29.7	1.3	75.6
Pi-PCM3-I	–	–	–	–	–	100	0.1	100
Pi-PCM3-II	–	–	–	10.9	21.0	68.1	0.2	90.7
Pi-PCM4-I	–	–	–	–	–	100	–	100
Pi-PCM4-II	–	–	–	–	45.6	54.4	–	92.8

–, not detectable; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose.

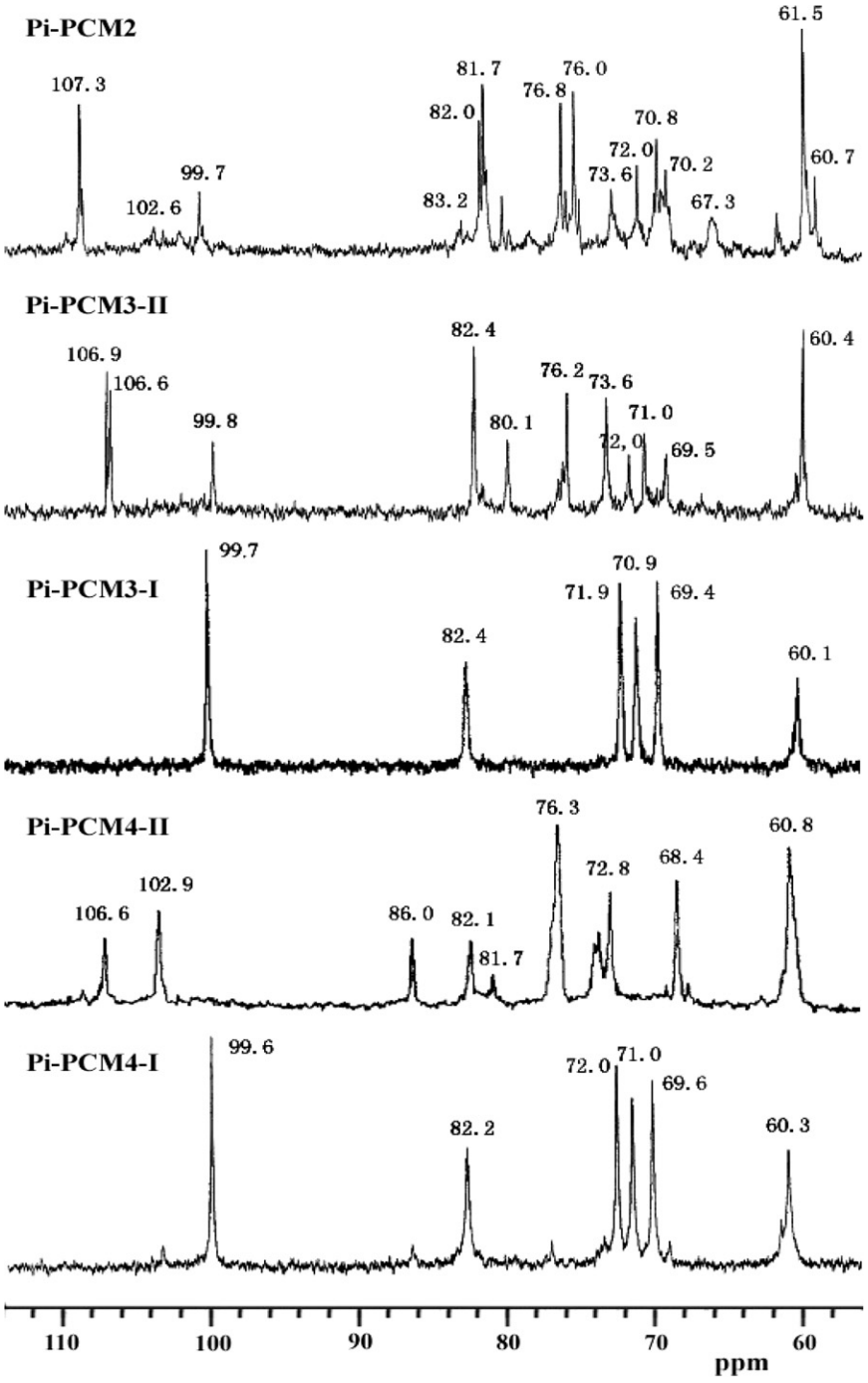


Fig. 2. The ^{13}C NMR spectra of Pi-PCM2, Pi-PCM3-II, Pi-PCM3-I, Pi-PCM4-II, and Pi-PCM4-I.

computing a classical Zimm plot from light scattering data collected at various angles (θ) for each polymer concentration (c). Fig. 3(a) and (b) show the Zimm plots for Pi-PCM0 in 0.2 M NaCl and for Pi-PCM4-I in 0.25 M LiCl/Me_2SO at 25 °C, respectively. Fig. 4(a) and (b) illustrate the angle dependences of Kc/R_θ for the water-soluble polysaccharides (Pi-PCM0 to Pi-PCM2) in 0.2 M NaCl and the water-insoluble polysaccharides (Pi-PCM3-I, -II, and Pi-PCM4-I, -II) in 0.25 M LiCl/Me_2SO at 25 °C, respectively. Here K was the light scattering constant and R_θ was the reduced Rayleigh ratio. The M_w, $\langle s^2\rangle_z^{1/2}$ and $[\eta]$ values of the polysaccharides as determined by LLS and viscometry measurements (Table 2) reveal that the M_w values of the mycelial polysaccharides increased from 6.46×10^4 to 4.36×10^6 in the sequential extraction, indicating that high molecular weight polysaccharides could be obtained by extraction with alkali and acid solution. In general, $[\eta]$ and $\langle s^2\rangle_z^{1/2}$ values are related to chain stiffness of polymer, and the relatively higher values of $[\eta]$ and $\langle s^2\rangle_z^{1/2}$ reflect a relatively more expanded chain.

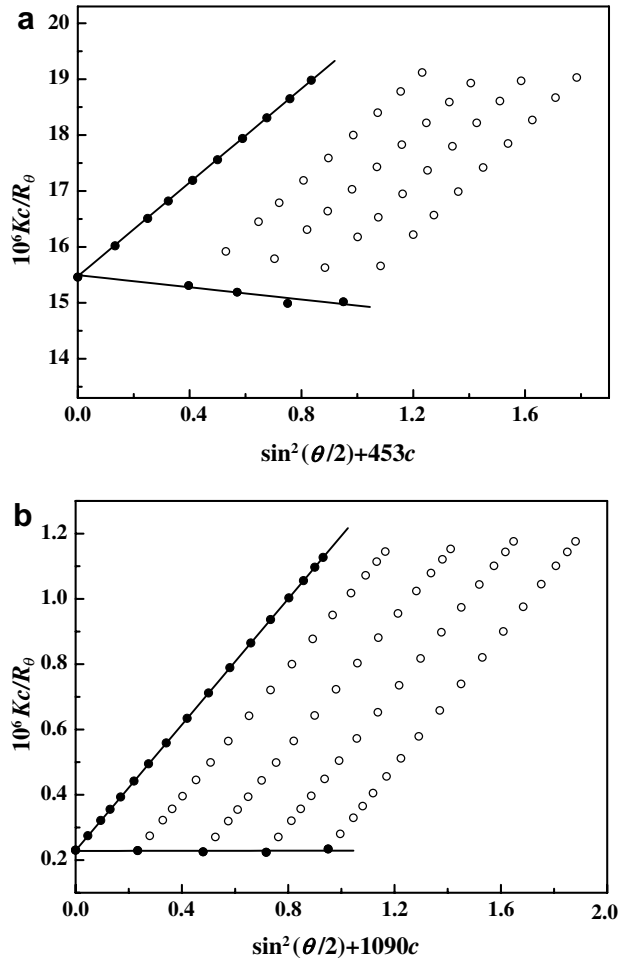


Fig. 3. Zimm plots for Pi-PCM0 in 0.2 M aqueous NaCl (a) and for Pi-PCM4-I in 0.25 M LiCl/Me_2SO (b) at 25 °C.

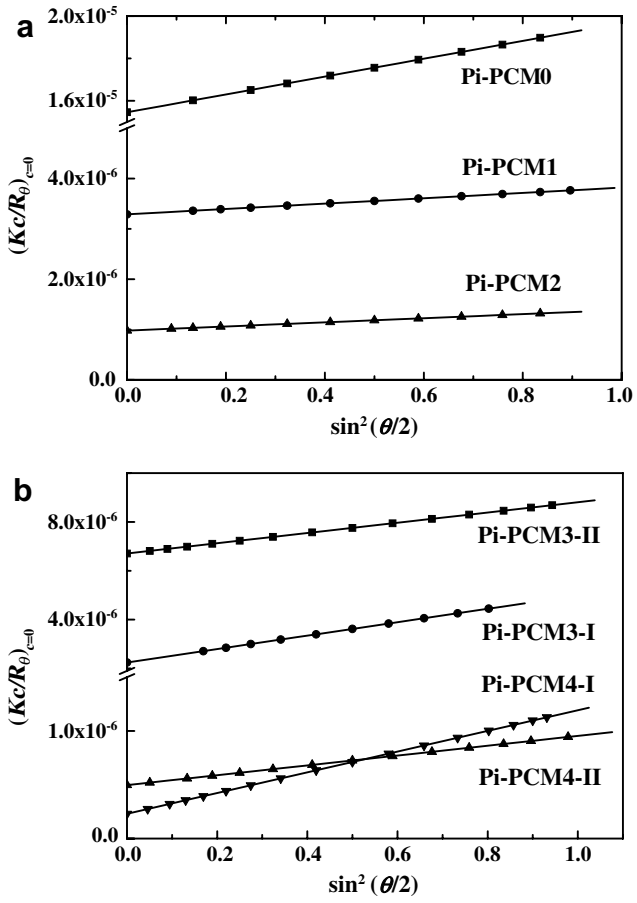


Fig. 4. The angle dependences of Kc/R_θ for water-soluble polysaccharides (Pi-PCM0 to Pi-PCM2) in 0.2 M aqueous NaCl (a) and water-insoluble polysaccharides (Pi-PCM3-I, -II, and Pi-PCM4-I, -II) in 0.25 M LiCl/Me_2SO (b) at 25 °C.

Compared with its relatively low M_w value, the relatively high $[\eta]$ of Pi-PCM3-I in 0.25 M LiCl/Me_2SO suggested an expanded flexible chain conformation, which was in line with the characteristics of (1 → 3)-α-D-glucan (Huang & Zhang, 2005). In contrast, Pi-PCM1 and Pi-PCM2 having high M_w with low $[\eta]$ existed as a compact random coil in aqueous solution, close to the global shape. In order to confirm the results, the Hester–Mitchell approach, which is a favorable method for molecules occupying a spherical volume in solution, was applied to the LLS and viscometry data. On the basis of the Hester–Mitchell method combined with Guth's modification of Einstein's viscosity relationship, the hydrodynamic radius (R_η) and the ratio of the geometric-to-hydrodynamic radii (ρ) can be determined as follows (Striegel, Plattner, & Willett, 1999):

$$R_\eta = [240/(\pi N_A)]^{1/3}(M[\eta])^{1/3}/2 \quad (5)$$

$$\rho = \langle s^2\rangle_z^{1/2}/R_\eta \quad (6)$$

Substituting the data of M_w, $\langle s^2\rangle_z^{1/2}$ and $[\eta]$ into Eqs. (5) and (6), the ρ value of Pi-PCM1 and Pi-PCM2 was found to be 0.70 and 0.71, respectively, which was comparable to the value for a homogeneous sphere (0.77) (Kubota, Fujishige, & Ando, 1990; Meewes, Rička, de Silva, Nyffenegger, & Binkert, 1991). These results further confirmed that Pi-PCM1 and Pi-PCM2 existed almost as a sphere in 0.2 M aqueous NaCl.

3.3. In vitro and in vivo antitumor activity

According to the *in vitro* inhibition ratio of HL-60 leukemia cells by the three heteropolysaccharides (Pi-PCM0, Pi-PCM1, and Pi-PCM2) at different concentrations (50, 100, and 200 μg/mL) (Fig. 5), all polysaccharides exhibited strong inhibition against cell growth at all concentrations, and there was also a dose–response relationship between concentration of the mycelial polysaccharides and suppression of HL-60 cell proliferation. In particular, the three water-soluble fractions presented significantly high inhibition ratio of more than 80% at the concentration of 200 μg/mL, suggesting that they had potential for investigation for leukemia therapy. It was noted that although extracellular polysaccharide Pi-PCM0 obtained by spay-drying suffered from high temperature, Pi-PCM0 showed

Table 2
The M_w, $\langle s^2\rangle_z^{1/2}$ and $[\eta]$ of all seven polysaccharide fractions from *Poria cocos* mycelia

Sample	Solvent	$[\eta]$ (mL g^{-1})	$M_w \times 10^{-4}$ (g mol^{-1})	$\langle s^2\rangle_z^{1/2}$ (nm)
Pi-PCM0	0.2 M NaCl	10.4	6.46	34.2
Pi-PCM1	0.2 M NaCl	11.0	30.4	26.3
Pi-PCM2	0.2 M NaCl	13.0	103	42.3
Pi-PCM3-II	0.25 M LiCl/Me_2SO	22.8	14.9	36.6
Pi-PCM3-I	0.25 M LiCl/Me_2SO	184	45.2	55.1
Pi-PCM4-II	0.25 M LiCl/Me_2SO	28.7	201	62.7
Pi-PCM4-I	0.25 M LiCl/Me_2SO	440	436	120.6

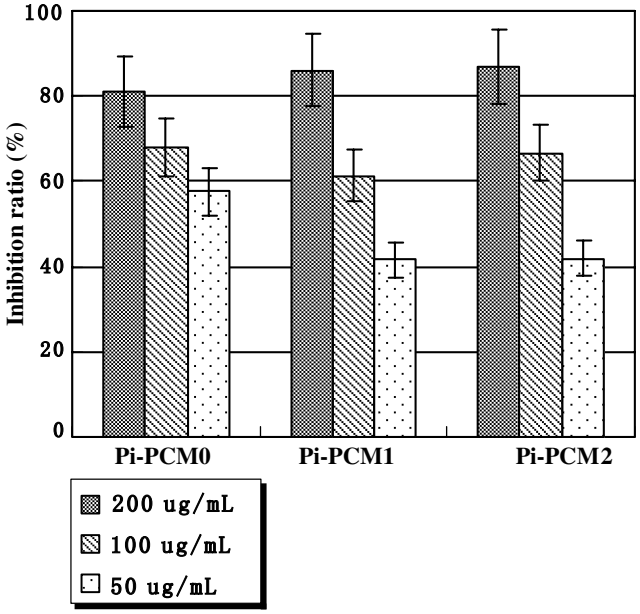


Fig. 5. The *in vitro* inhibition ratio of HL-60 leukemia cell by Pi-PCM0, Pi-PCM1, and Pi-PCM2 at different concentration.

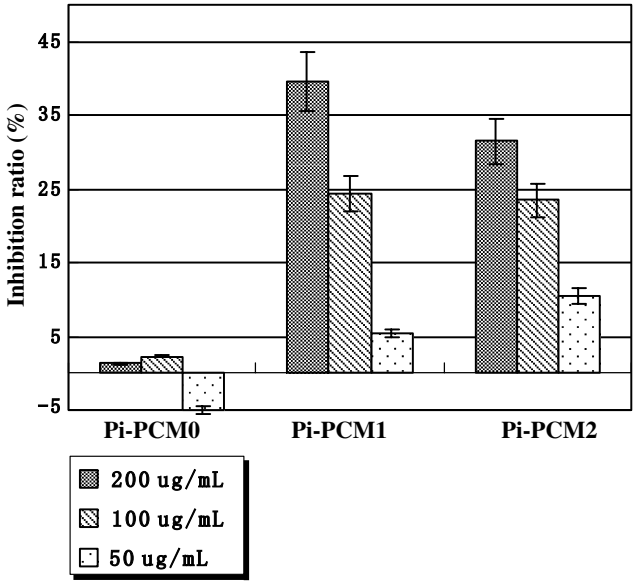


Fig. 6. The inhibition ratio of *in vitro* human MCF-7 cell growth by Pi-PCM0, Pi-PCM1, and Pi-PCM2 at different concentration.

high inhibition ratios of 57.6%, 68.1% and 81.0% at the concentration of 50, 100, and 200 μg/mL, respectively. This indicated that high temperature during spay-drying had no effect on its antitumor activity. Although in the *in vitro* inhibition ratio of human MCF-7 cell growth by Pi-PCM0, Pi-PCM1, and Pi-PCM2 at different concentration (50, 100, and 200 μg/mL) (Fig. 6), the three heteropolysaccharides exhibited a relatively lower inhibition to MCF-7 cell than HL-60 leukemia cell, Pi-PCM1 and Pi-PCM2 had an inhibition ratio of 30-40% at the concentration of 200 μg/mL. No antiproliferation effect of these polysaccharides on monkey kidney cells (Vero) was observed (data not shown), implying that they had no direct cytotoxicity to non-cancerous cells. On the basis of *in vitro* proliferation assays for suspended HL-60 cell and adherent MCF-7 cell, it was concluded that the three water-soluble polysaccharides were potent tumor cell growth inhibitors that showed selectively higher antitumor activities against suspended cells than adherent ones.

In the results of the *in vivo* antitumor activities of the three heteropolysaccharides against Sarcoma 180 solid tumor (Table 3) all the heteropolysaccharides having M_w ranged from 6.46×10^4 to 103×10^4 exhibited potent *in vivo* inhibition ratio of more than 40%. Compared with other polysaccharides, Pi-PCM2 having a high M_w showed a stronger inhibition ratio of 54.4%. The antitumor activity of high molecular weight polysaccharides is considered to be a consequence of stimulation of the immune response in the host, rather than direct killing tumor cells, as reported by Wasser (2002) and Zjawiony (2004).

Table 3
The results of the *in vivo* antitumor activities of polysaccharide fractions Pi-PCM0, Pi-PCM1, and Pi-PCM2 against Sarcoma 180 solid tumor

Sample	$M_w \times 10^{-4}$ (g mol^{-1})	Dose (mg/kg × days)	Inhibition ratio, ξ (%)	Complete repression
Pi-PCM0	6.46	20 × 10	42.1	0/8
Pi-PCM1	30.4	20 × 10	40.3*	0/8
Pi-PCM2	103	20 × 10	54.4*	0/8

* $p < 0.05$.

4. Conclusions

From *P. cocos* mycelia derived from a pilot-scale 50-L fermenter by submerged cultivation biotechnology, six polysaccharide fractions were isolated sequentially by a

solvent extraction system. In addition, the extracellular polysaccharide (Pi-PCM0) produced by the mycelia was obtained from the culture media by spray-drying. The water-soluble fractions were heteropolysaccharides composed of glucose, galactose, and mannose, whereas the water-insoluble Pi-PCM3-I and Pi-PCM4-I were (1 → 3)-α-D-glucans. The results from LLS accompanied with viscometry revealed that Pi-PCM1 and Pi-PCM2 existed as a compact random coil in aqueous solution, close to a spherical shape, while Pi-PCM3-I exhibited a relatively expanded flexible chain in 0.25 M LiCl/Me_2SO. Notably, the water-soluble polysaccharides (Pi-PCM0 to Pi-PCM2) all exhibited strong antitumor activities against Sarcoma 180 solid tumor implanted in BALB/c mice *in vivo* and against HL-60 tumor cell *in vitro*.

Acknowledgements

This work was supported by major grant of the National Science Foundation of China (30530850), and UGC-AoE Plant and Fungal Biotechnology Project of Hong Kong.

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