

COMPARISON OF POLYSACCHARIDES ISOLATED FROM THE MYCELIA OF A CULTIVATED STRAIN OF *PORIA COCOS* GROWN IN DIFFERENT LIQUID CULTURE MEDIA*

Li-na Zhang^{a**}, Li Chen^a, Yong Jin^a, Yu-lu Lin^b, Peter Chi Keung Cheung^c and Li-guo Chen^d

^a Department of Chemistry, Wuhan University, Wuhan 430072, China

^b College of Chemistry and environment engineering, Jiangnan University, Wuhan 430056, China

^c Department of Biology, The Chinese University of Hong Kong, Hong Kong, China

^d Fungus Institute, Huazhong University of Agriculture, Wuhan 430070, China

Abstract Mycelium of a cultivated strain of *Poria cocos* was grown by submerged fermentation in a liquid medium containing corn steep liquor with orbital shaking. Six polysaccharides coded as ac-PCM1, ac-PCM2, ac-PCM3-I and II, ac-PCM4-I and II were isolated from the mycelium by extracting with 0.9% NaCl aqueous solution, hot water, 0.5 mol/L NaOH aqueous solution and 88% formic acid. Exo-polysaccharide was obtained from the culture medium and coded as ac-PCM0. The monosaccharide composition and molecular weights of these polysaccharides were characterized by using infrared spectroscopy, gas chromatography, elemental analysis, ¹³C-NMR, viscometry and light scattering. The results indicated that ac-PCM0, ac-PCM1 and ac-PCM2 are heteropolysaccharides containing glucose, galactose, mannose and fucose, and ac-PCM3-I and ac-PCM3-II mainly consist of *D*-glucose. The content of the glucose in the polysaccharides increased with the isolation progress. Remarkably, α -glucan and β -glucan coexisted in the extract by NaOH aqueous solution (ac-PCM3), and could be separated by chemical methods. The protein in the ac-PCM polysaccharides cultured from the medium containing corn steep liquor was higher than that in the ab-PCM from the medium with bran extract. Therefore, the polysaccharides from *Poria cocos* mycelia cultured in different media have different chemical composition, molecular weights and conformations.

Keywords *Poria cocos* mycelium, Culture medium, Polysaccharide, Monosaccharide composition, Molecular weight

INTRODUCTION

Polysaccharides are being increasingly identified with the processes of biological recognition, regulation and information storage^[1] and also play an important role in the structural composition of living systems^[2]. In the last few decades, the biological activities and solution properties^[3, 4] of polysaccharides have attracted more and more attention in biochemistry and medicine. *Poria cocos* has long been used as traditional Chinese medicine and is composed mainly of a polysaccharide named pachyman. Takada *et al.*^[5] first indicated that pachyman only contains glucose residues, and then Warsi *et al.*^[6] reported that pachyman is a polymer of β -glucose, containing 1,3-linkages. Hoffman *et al.*^[7] concluded that pachyman contains little 1,6-linked branching and has a molecular weight of 19.4×10^4 . It has been found that polysaccharide produced by cultivation mainly contains β -(1→3)-*D*-glucan with few branches, and is almost identical with that prepared from naturally occurring sclerotium of *Poria cocos* and has a molecular weight of 3.7×10^5 ^[8]. Despite all these researches, there are very few reports that deal with the chemical compositions, molecular weights and chain conformations of different polysaccharides isolated

* This work was supported by the National Natural Science Foundation of China (20074025), the Area of Excellence (AoE) on Plant and Fungal Biotechnology Project of the Hong Kong Government, and Key Laboratory of Cellulose and Lignocellulosics Chemistry of Chinese Academy of Sciences.

** Corresponding author: Li-na Zhang (张俐娜), E-mail: lnzhang@public.wh.hb.cn

Received September 27, 2002; Revised January 3, 2003; Accepted January 14, 2003

from sclerotium and mycelium of *Poria cocos* in detail.

We have isolated three heteropolysaccharides (PC1, PC2, PC2-A) and two β -D-glucans (PC3, PC4) from a fresh sclerotium of *Poria cocos* by sequential extraction using 0.9% NaCl aqueous solution, hot water, 0.5 mol/L NaOH and 88% formic acid previously^[9]. Among them, PC3 (a linear β -(1 $\rightarrow$ 3)-D-glucan) and PC4 (a β -(1 $\rightarrow$ 3)-D-glucan with a few branches and glucuronic acid) were the major fractions of the *Poria cocos* polysaccharides. It has been reported that the structure of fungal polysaccharides depends on the fungal species, the conditions of growth and the extraction method^[10]. In our recent work^[11], six polysaccharides have been isolated from the *Poria cocos* mycelia produced from a cultivated strain (No. 5 $\cdot$ 78) in a medium with bran extract by using extraction similar to that mentioned above^[9], and their structure and molecular weights were characterized by infrared (IR) spectroscopy, gas chromatography (GC), ¹³C-NMR, light-scattering and viscometry^[11]. In this work, we attempted to investigate the influence of different culture media on the chemical composition and molecular weight of polysaccharides isolated from *Poria cocos* mycelium.

EXPERIMENTAL

Cultivation of Poria cocos Mycelia

The cultivation of *Poria cocos* mycelia was performed in Huazhong University of Agriculture in China. The fungal hyphae coded as 5 $\cdot$ 78, from a cultivated strain of *Poria cocos*, were obtained from the Institute of Microbiology, the Chinese Academy of Science. The fungi were inoculated and incubated in a culture medium, which consisted of D-glucose (25 g), yeast extract (3.2 g), corn steep liquor (7 mL), KH₂PO₄ (1 g), CaCl₂ $\cdot$ 2H₂O (0.06 g), MnCl₂ $\cdot$ 4H₂O (5 mg), MgSO₄ $\cdot$ 7H₂O (0.5 g), ZnCl₂ (4 mg), Fe₂(SO₄)₃ (5 mg), Vitamin B₁ (0.1 mg) and water (1 L), under a constant temperature of 25°C for 13 days with orbital shaking. Light-brown mycelia were harvested by filtration through gauze, washed with water 5 times, and freeze-dried by a lyophilizer (Christ, Alpha 1–2, Germany). The powder of dry *Poria cocos* mycelia was obtained by milling. In addition, the supernatant from the culture medium was also collected to obtain the exo-polysaccharide.

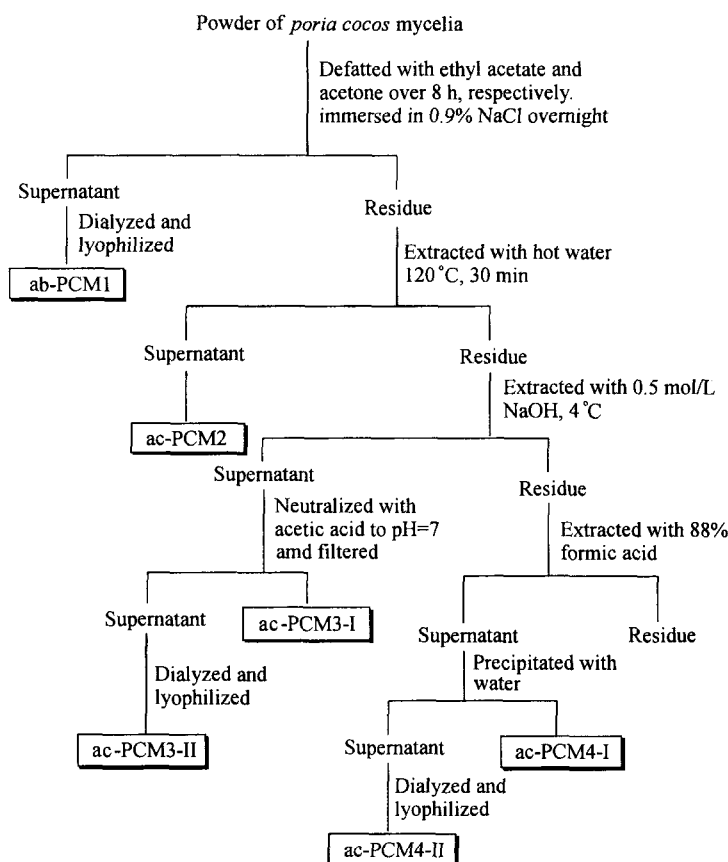
Isolation and Purification of Polysaccharides

The *Poria cocos* mycelia were firstly defatted in a Soxhlet extractor using ethyl acetate and acetone as extractants for 8 h, respectively. The resulting residues were soaked in 0.9% NaCl aqueous solution overnight, then homogenized. The supernatant containing the mycelial polysaccharide was centrifuged, followed by dialysis and lyophilization to obtain first polysaccharide coded as ac-PCM1. The residues were sequentially extracted by using the extraction procedure outlined in Scheme 1.

Each polysaccharide was purified according to the previous method^[12]. In brief, the polysaccharides were decolorized and purified by using 30% H₂O₂ and the Sevag method, followed by dialysis against water for 4 days and distilling water for 3 days to remove small molecule impurities. The resulting mixtures containing the polysaccharides were concentrated in a rotary evaporator under diminished pressure below 50 °C, and then lyophilized to obtain yellow products (ac-PCM0, ac-PCM1, ac-PCM2, ac-PCM3-II and ac-PCM4-II) and brown products (ac-PCM3-I and ac-PCM4-I), respectively. Exo-polysaccharide coded as ac-PCM0 was purified similarly.

Characterization of Chemical Structure

The UV spectra of all polysaccharides were measured by a UV spectrophotometer (UV-160 Shimadzu, Japan). The degree of purity of the polysaccharides was assessed by the UV absorption peak at 200 nm for polysaccharide and the absorption peaks at 280 nm and 600 nm for protein and pigments, respectively. IR spectra of the polysaccharides pressed into KBr pellets were recorded with a Nicolet FT-IR (360 FT-IR ESP, Thermo Nicolet, Avatar) spectrometer within the range of 400 to 4000 cm⁻¹. Protein content was measured by the Kjeltec1030 self-analyzer (Switzerland) according to the semi-micro Kjeldahl principle. The N element content was measured by an elemental analyzer (Heraeus Co., Germany).



Scheme 1 Extraction of the ac-PCM polysaccharides from *Poria cocos* mycelia

^{13}C -NMR spectrum was recorded on an Avance DRX-400 spectrometer (Bruker Co. Germany/Switzerland) at 25°C, with DSS and TMS as the internal standards. D_2O was used as the solvent for ac-PCM1, and $\text{DMSO}-d_6$ for ac-PCM3-I and ac-PCM4-II.

The monosaccharides in the samples from *Poria cocos* mycelia were measured by GC as alditol acetate derivative^[13]. The sample (15 mg) was transferred into a small screw-capped vial to which concentrated sulphuric acid (0.7 mL, 12 mol/L) was added, and the suspension was dispersed by continuous stirring with a magnetic bar at 35°C for 60 min. Subsequently, distilled water (3.0 mL) was added, and then the mixture was vortex-mixed and heated in a boiling water bath with shaking for 60 min before being cooled under tap-water to room temperature. An aliquot portion of the acid hydrolysate (3.0 mL) was transferred to a test-tube cooled in an ice bath and an internal standard, allose (1 mL, 1 mg/mL) was added. Ammonia (1.25 mL, 12 mol/L) was added to the test-tube to neutralize the acid hydrolysate. Octan-2-ol (5 μL) and freshly prepared NaBH_4 (0.2 mL, 200 mg/mL of 2 mol/L NH_3) were added, then mixed and kept at 40°C for 30 min before adding 0.4 mL glacial acetic acid. An aliquot portion of the resulting mixture (0.2 mL) was transferred into a new test-tube containing 0.3 mL 1-methylimidazole and 2.0 mL acetic anhydride. The mixture was left at room temperature for 10 min before adding 5.0 mL distilled water and cooled under tap water to room temperature. Dichloromethane (1.0 mL) was added to the sample and vortex-mixed. The top layer was removed and the bottom layer was washed with distilled water 3 times and dried with anhydrous Na_2SO_4 , and then stored in a capped vial at -20°C before GC analysis. The derived sugars in the sample and standards were analyzed by using an HP 6890 gas chromatograph (Hewlett Packard, U.S.A) equipped with flame ionization detector and an Alltech DB-225 capillary column (15 m $\times$ 0.25 mm, 0.25 μm film thickness) with the following oven temperature program: initial temperature,

180°C with a heating rate of 4 K/min to 220°C and held at 220°C for 30 min. The injection sample volume was 2 μL , the carrier gas was helium.

Viscosity Measurement

The viscosity of the polysaccharide solutions was measured at $30 \pm 0.05^\circ\text{C}$ with an Ubbelodhe capillary viscometer. A 0.2 mol/L NaCl aqueous solution and a 0.25 mol/L LiCl/DMSO were used as solvents for samples ac-PCM0, ac-PCM1, ac-PCM2, and ac-PCM3-I, ac-PCM3-II, ac-PCM4-II, respectively. The kinetic energy correction was assumed negligible. Huggins and Kraemer plots were used to estimate the intrinsic viscosity $[\eta]$.

Laser Light Scattering

The light-scattering intensity was measured with a multi-angle laser light scattering (MALLS) instrument (DAWN[®] DSP, Wyatt Technology Co. USA) at 633 nm in an angular range from 23 to 147° at 25°C. Samples ac-PCM0, ac-PCM1 and ac-PCM2 were dissolved in 0.2 mol/L NaCl aqueous solution for 24h to obtain clear aqueous solutions. Sample solutions for ac-PCM3-I, ac-PCM3-II and ac-PCM4-II were prepared in 0.25 mol/L LiCl/DMSO. Optical clarification of the solution was performed with subsequent filtration through a 0.45 μm Millipore filter (Whatman, England) into the scattering cell (SV mode). The refractive index increments (dn/dc) at 633 nm and 25°C were found to be 0.140 $\text{g}^{-1} \text{cm}^3$ in 0.2 mol/L NaCl aqueous solution and 0.062 $\text{g}^{-1} \text{cm}^3$ in 0.25 mol/L LiCl/DMSO solution, respectively.

SEC-MALLS Measurements

Size exclusion chromatography (SEC) integrated with multi-angle static light scattering (SEC-LLS) is convenient for the measurement of absolute molecular weight and its distribution without the aid of standard samples. The SEC-LLS measurements of the polysaccharides were performed on the above MALLS photometer combined with a P100 pump (Thermo Separation Products) equipped with TSK-GEL G6000 PWXL combined with either a G4000 PWXL column (7.8 mm $\times$ 300 mm) for aqueous solution or a G4000H6 column (7.5 mm $\times$ 300 mm) for 0.25 mol/L LiCl/DMSO solution, together with a differential refractive index detector (OPTILAB DSP). The eluent was 0.2 mol/L NaCl aqueous solution and 0.25 mol/L LiCl/DMSO solution with a flow rate of 1.0 mL/min, respectively. All the solutions used were filtered with a sand filter, and then with a 0.45 μm filter (CA, Puradisc[™] 13 mm Syringe Filters, Whatman, England) for 0.2 mol/L NaCl aqueous solution and with a 0.45 μm filter (PTFE, Puradisc[™] 13mm Syringe Filters, Whatman, England) for 0.25 mol/L LiCl/DMSO. Astra software was utilized for data acquisition and analysis.

RESULTS AND DISCUSSION

The IR spectra of seven polysaccharide fractions from *Poria cocos* are shown in Fig. 1. All samples have the characteristic absorption peaks of polysaccharide near 1245 and 1650 cm^{-1} . ac-PCM3-II, ac-PCM4-I and ac-PCM4-II have an absorption peaks at 890 cm^{-1} , which is the characteristic band of β -D-glucan. However, ac-PCM3-I that has been extracted with 0.5 mol/L NaOH aqueous solutions shows two absorption peaks at 847 and 933 cm^{-1} , indicating the presence of α -D-glucan. Moreover, ac-PCM0 exhibits the characteristic absorption peaks of mannose at 808 and 872 cm^{-1} .

The monosaccharide profiles of the polysaccharides from GC analysis are shown in Fig. 2, and their monosaccharide composition are summarized in Table 1. The results indicate that ac-PCM3-I and ac-PCM3-II mainly consist of D-glucose, while ac-PCM0, ac-PCM1 and ac-PCM2 contain glucose, galactose, mannose and fucose. The content of the glucose in the polysaccharides increases with the process of the extraction. On the whole, the results suggest that the solubility of β -glucan is lower than that of the heteropolysaccharides.

The ¹³C-NMR spectra of ac-PCM3-I and ac-PCM4-II in DMSO-*d*₆ solvent are shown in Fig. 3. The chemical shifts of the main chain are summarized in Table 2. The signals of ac-PCM3-I at $\delta = 99.9, 78.7, 73.5, 72.0, 70.9$ and 60.9 are assigned to C1, C3, C5, C2, C4 and C6 of the α -(1 $\rightarrow$ 3)-D-glucan main chain, and those at $\delta = 102.1, 82.9, 76.8$ and 65.8 are assigned to C1', C3', C5' and C6' of the 1,6-linked glucan side chain^[14]. The peak at $\delta = 69.5$ is the substituted C6, signal of the main chain. The resonance peaks at $\delta = 172\text{--}175$ are

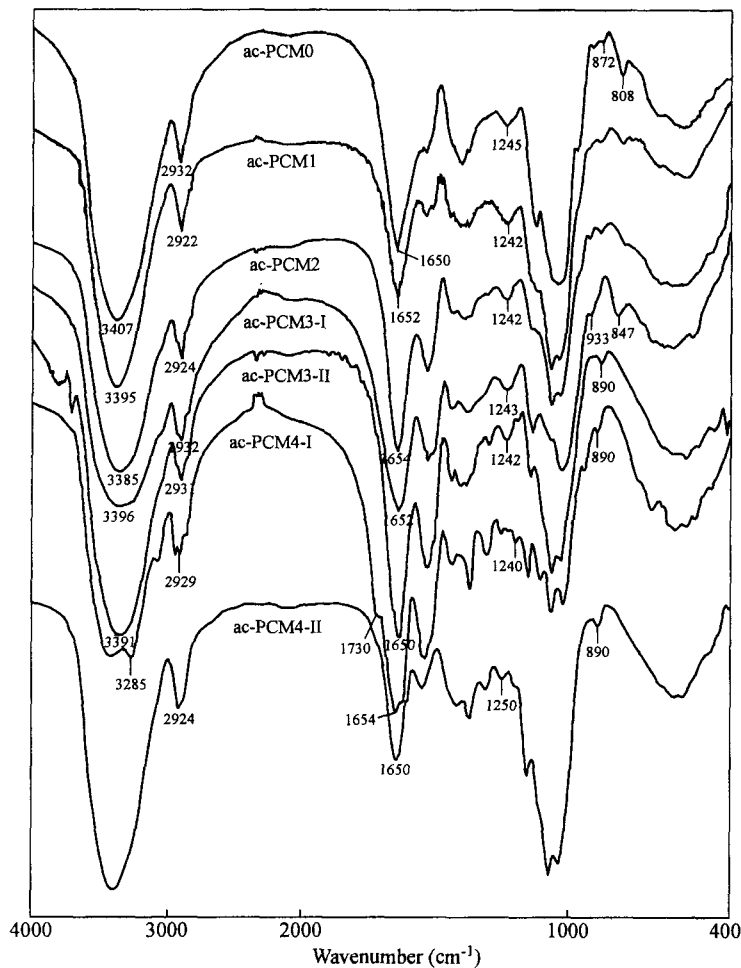


Fig. 1 FT-IR spectra of polysaccharides from *Poria cocos* mycelia

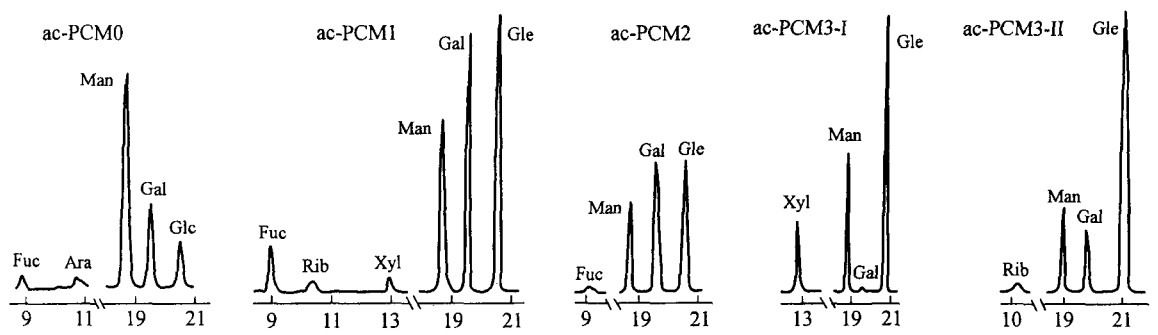


Fig. 2 GC traces of the alditol acetate derivatives of hydrolyzed polysaccharide of ac-PCM

assigned to the $\text{—CONH—}^{[15]}$ group of protein. This indicates that ac-PCM3-I is composed of a protein bound 1,3-linked α -D-glucan backbone with 1,6-linked glucan branched chain. In the ^{13}C -NMR spectrum of ac-PCM4-II, the strong signals at $\delta = 103.0, 86.2, 76.3, 72.8, 68.4$ and 60.9 are assigned to C1, C3, C5, C2, C4 and C6 of β -(1 $\rightarrow$ 3)-D-glucan main chain, with the small peak at $\delta = 70.0$ as the substituted C6_s of the β -(1 $\rightarrow$ 3)-D-glucan

main chain. These results are in agreement with the data obtained from GC, IR and protein analysis. Figure 4 shows the ¹³C-NMR spectra for ab-PCM2-I and ab-PCM3-I from *Poria cocos* mycelia cultured in the medium containing bran^[11]. From the above results, both ab-PCM3-I and ac-PCM3-I seem to be branched α-(1→3)-D-glucan, with the former one having less branching than the latter. However, ab-PCM2-I exhibits the characteristic signals of a linear α-(1→3)-D-glucan. In addition, the protein in ac-PCM polysaccharides cultured from the medium containing corn steep liquor is higher than that in the ab-PCM from the media with bran (Table 1). It is believed that the protein is bound with the polysaccharide, because the procedure of the Sevag method was repeated six times to remove any free protein.

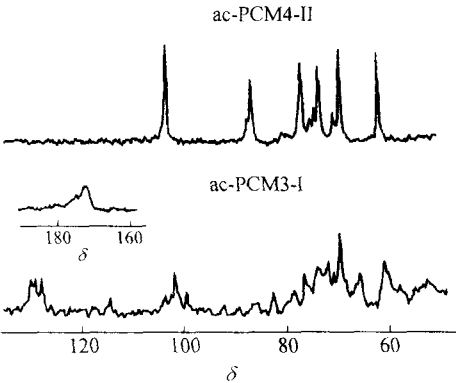


Fig. 3 ¹³C-NMR spectra of ac-PCM3-I and ac-PCM4-II in DMSO-*d*₆ at 25°C

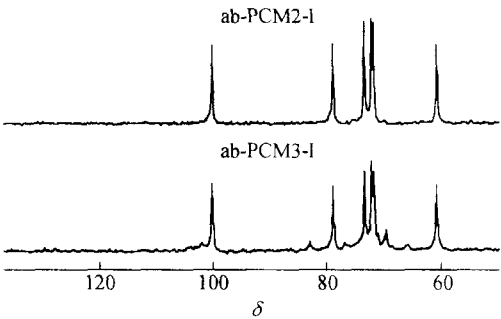


Fig. 4 ¹³C-NMR spectra of ab-PCM2-I and ab-PCM3-I in DMSO-*d*₆ solvent at 25°C^[11]

Table 1. Chemical composition of polysaccharides from *Poria cocos* mycelia

Sample	Content (%)						Protein (%)	Yield (%)
	Fuc	Ara	Xyl	Man	Gal	Glc		
ac-PCM0	1.4	1.0	–	43.0	27.4	27.2	25.5	
ac-PCM1	4.5	–	+	15.8	23.9	53.4	42.9	1.7
ac-PCM2	0.8	–	–	19.1	29.7	51.4	18.1	1.7
ac-PCM3-I	–	–	4.6	13.3	+	81.6	39.4	9.3
ac-PCM3-II	–	–	–	2.2	1.7	95.9	40.1	2.4
ac-PCM4-I								4.1
ac-PCM4-II							14.2	1.3

–: Not detected; +: Trace amount
Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose

Table 2. Comparison of ¹³C NMR chemical shifts for the polysaccharides in the solution

Sample	Solvent	Chemical shift (δ)							Source
		C1	C2	C3	C4	C5	C6	C6 _s	
α-(1→3)-D-glucan	Deuterium oxide	100.3	71.4	81.7	70.8	72.7	61.5		Ref. [16]
α-(1→3)-D-glucan	LiCl/DMSO- <i>d</i> ₆	99.3	70.9	82.2	69.5	71.9	60.4		Ref. [17]
ab-PCM2-I	DMSO- <i>d</i> ₆	100.1	71.9	78.7	71.6	73.2	60.5		Ref. [11]
ab-PCM3-I	DMSO- <i>d</i> ₆	100.1	72.0	78.8	71.6	73.3	60.5	66.3	Ref. [11]
ac-PCM3-I	DMSO- <i>d</i> ₆	99.9	72.0	78.7	70.9	73.5	60.9	65.8	This work
β-(1→3)-D-glucan	DMSO- <i>d</i> ₆	103.0	72.8	86.2	68.4	76.3	60.9		Ref. [18]
ac-PCM4-II	DMSO- <i>d</i> ₆	103.0	72.8	86.2	68.4	76.3	60.9	70.0	This work

The Zimm plot of ac-PCM4-II in 0.25 mol/L LiCl/DMSO at 30°C is shown in Fig. 5. The measured weight-average molecular weight M_w and intrinsic viscosity $[\eta]$ of all samples in 0.2 mol/L NaCl aqueous solutions or 0.25 mol/L LiCl/DMSO are summarized in Table 3. On the whole, the M_w of ac-PCM polysaccharides increases progressively along the extraction scheme. Interestingly, ac-PCM3-I and ab-PCM3-I have similar M_w but different $[\eta]$. This might be explained by the fact that ac-PCM3-I contains much more protein and branching than ab-PCM3-I, resulting in a compact-coil conformation in the solution, thus leading to a reduction of $[\eta]$. As shown in Table 3, the M_w for PCM polysaccharides obtained from mycelium grown in media containing corn steep liquor and bran are different, indicating that the chemical composition of the media also affects the molecular weight and chain conformation.

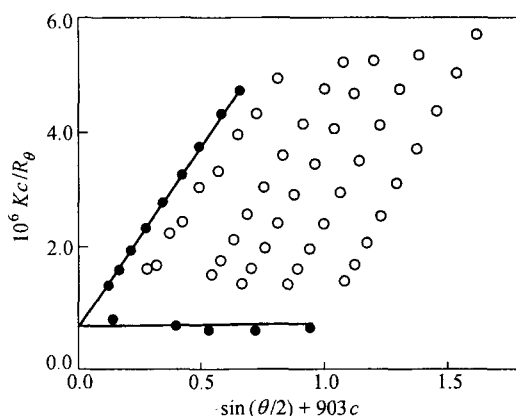


Fig. 5 Zimm plot for ac-PCM4-II in 0.25 mol/L LiCl/DMSO at 30°C

Table 3. Experimental results of $[\eta]$ and M_w of the polysaccharides in different solvents at 30°C

Sample	Solvent	$[\eta]$ (cm ³ g ⁻¹)	$M_w \times 10^{-4}$	Source
ac-PCM0	0.2 mol/L NaCl	8.0	10.1	This work
ac-PCM1	0.2 mol/L NaCl	10.2	12.5	
ac-PCM2	0.2 mol/L NaCl	19.8	17.0 ^a	
ac-PCM3-I	0.25 mol/L LiCl/DMSO	43.8	58.1	
ac-PCM3-II	0.25 mol/L LiCl/DMSO	50.3	31.8	
ac-PCM4-II	0.25 mol/L LiCl/DMSO	114.9	171	
ab-PCM0	0.5 mol/L NaCl	4.4	9.3	Ref. [11]
ab-PCM1	0.5 mol/L NaCl	29.3	—	
ab-PCM2-I	0.25 mol/L LiCl/DMSO	12.0	5.6	
ab-PCM3-I	0.25 mol/L LiCl/DMSO	124.6	65.2	
ab-PCM3-II	0.25 mol/L LiCl/DMSO	11.9	13.0	
ab-PCM4-II	0.25 mol/L LiCl/DMSO	39.3	25.5	

^aData obtained from SEC-LLS

Therefore, the bran and corn steep liquor in the affected significantly media the chemical structure, molecular weight and conformation of the polysaccharides from *Poria cocos* mycelia. Studies on the bioactivities of these mycelial polysaccharide fractions are underway.

REFERENCES

- 1 Dwek, R.A., Chem Rev, 1996, 96:683
- 2 Brant, D., Current Opinion in Structure Biology, 1999, 9: 556
- 3 Vincent, E.C. and Liu, F., Current Medicinal Chemistry, 1999, 6: 1

- 4 Jeon, K.J., Katsuraya, K., Kaneko, Y., Mimura, T. and Uryu, T., *Macromolecules*, 1997, 30: 1997
- 5 Takada, K., *Med. Tottori Agric. Coll.*, 1935, 3: 1
- 6 Warsi, S.A. and Whelan, W.J., *Chem. Ind.*, 1957, 1573
- 7 Hoffman, G.C., Simson, B.W. and Timell, T.E., *Carbohydr. Res.*, 1971, 20(11): 185
- 8 Narui, T., Takakashi, K., Kobayashi, M. and Shibata, S., *Carbohydr. Res.*, 1980, 87: 161
- 9 Zhang, L.N., Ding, Q., Zhang, P.Y. and Feng, H.Q., *Chem. J. Chinese Univ.*, 1997, 6: 990
- 10 Srivastava, R. and Kulshreshtha, D., *Phytochemistry*, 1989, 28: 2877
- 11 Lin, Y.L., Zhang, L.N., Jin, Y., Chen, L., Zhang, M., Han, H.W., Zhang, Z.Q. and Chen, L.G., *Acta Polymerica Sinica* (in Chinese), 2003, (1): 97
- 12 Zhang, L.N., Yang, L.Q., Ding, Q. and Chen, X.F., *Carbohydr. Res.*, 1995, 270: 1
- 13 Englyst, H.N., Quigley, M.F. and Hudson, G.J., *Analyst*, 1994, 119: 1497
- 14 Gorin, P.A.J., *Advances in Carbohydrate Chemistry and Biochemistry*, 1981, 38: 14
- 15 Andrzej, T., Romana, A. and Iwona, W., *Carbohydr. Res.*, 2001, 334: 71
- 16 Jansson, P., Kenne, L. and Schweda, E., *J. Chem. Soc. Perkin Trans. I*, 1988, 2729
- 17 Chen, J.H., Zhou, J.P., Zhang, L.N., Nakamura, Y. and Norisuye, T., *Polymer Journal*, 1998, 30(10): 838
- 18 Kogan, G., Alföldi, J. and Masler, L., *Biopolymers*, 1988, 27: 1055