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Note

Antitumor activities of heteropolysaccharides of *Poria cocos* mycelia from different strains and culture media

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Abstract

Ten water-soluble heteropolysaccharide fractions were isolated from *Poria cocos* mycelia cultured from one wild and one cultivated strain in two identical culture media differing only in one component: either corn steep liquor or bran extract. The chemical compositions, including monosaccharide profile, protein content, and molecular mass M_w of the mycelial polysaccharides were determined. Both the in vitro and in vivo antitumor activities of the heteropolysaccharides were evaluated and compared. The heteropolysaccharides from *Poria cocos* mycelia cultured with the wild strain in a medium containing corn steep liquor exhibited the highest antitumor activities against Sarcoma 180 in vivo.

Keywords: *Poria cocos* mycelia; Heteropolysaccharide; Antitumor activity; Protein-bound α-glucan; Correlation of structure to bioactivity

A number of fungal polysaccharides with antitumor activities, such as lentinan from *Lentinus edodes*,[1,2] grifolan from *Grifola frondosa*,[3,4] schizophyllan from *Schizophyllum commune*[5,6] and derivative of pachyman from *Poria cocos*[7] have attracted much attention. The antitumor activity of the fungal products is believed to be a consequence of stimulation of the cell-mediated immune response.[1,3] Chihara has demonstrated from in vivo murine models that the antitumor activity of mushroom polysaccharides is mediated by the host immune system rather than a direct cytotoxic action.[1] However, much controversy surrounds the biochemical and molecular principles of both the immunostimulation and the antitumor activity.[8] For instance, important structural requirements in connection with antitumoral effects, appear to be a triple helical, β-(1→6)-branched β-(1→3)-D-glucan with high molar mass.[9,10] In contrast, Saito and co-workers[11] and Gomaa and coworkers[12] described a single helix as a requirement for this activity. Demleitner and co-workers[13] have indicated that the β-(1→3)-glycosidic linkage is the essential structural feature for immunostimulatory and antitumoral effects, and no high molar mass is required. However, there are some antitumor polysaccharides with other chemical structures, such as hetero-β-glucan,[7] heteroglycan,[14] α-glucan[15] and α-glucan-protein[16] etc.

It has been demonstrated that the polysaccharide extracted from *Poria cocos* mycelium with water or aqueous alkali had anticarcinogenicity against Sarcoma 180 ascites tumor cells.[17] However, the effects of the culture media and strain species on the bioactivities of the heteropolysaccharides and α-glucan from *Poria cocos* mycelium have not yet been studied. In our previous work,[18–20] polysaccharide fractions have been isolated from the *Poria cocos* mycelia and its culture medium. The heteropolysaccharides were water-soluble and their monosaccharide compositions and protein content are summarized in Table 1. On the whole, the polysaccharides wc-PCM0, wc-PCM1, wc-PCM2, ac-PCM0, ac-PCM1, and ac-PCM2 obtained from the medium contained corn steep liquor had more protein,

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Table 1
Monosaccharide composition, protein content and yield of the heteropolysaccharides from *Poria cocos* mycelia

Sample	[6]Monosaccharide content in polysaccharaide (%)					Protein (%)	Yield (%)	Source (%)
	Fuc	Ara	Xyl	Man	Gal	Glc			
wc-PCM0	4.1	3.0	2.5	61.7	15.0	13.7	19.0		Ref. 18
wc-PCM1	10.5	–	–	24.5	27.5	37.5	30.6	1.8	
wc-PCM2	3.4	–	–	12.5	13.4	70.7	29.5	3.1	
wb-PCM0	–	6.1	3.9	11.4	5.9	71.7	12.8		Ref. 18
wb-PCM1	–	–	–	7.7	19.2	73.1	7.6	1.3	
wb-PCM2	–	+	+	0.9	1.3	95.9	8.5	2.0	
ac-PCM0	1.4	1.0	–	43.0	27.4	27.2	25.0		Ref. 19
ac-PCM1	4.5	–	+	15.8	23.9	53.4	42.9	1.7	
ac-PCM2	0.8	–	–	19.1	29.7	51.4	18.3	1.7	
ab-PCM0	–	9.2	11.1	21.5	12.7	45.4	11.6		Ref. 20
ab-PCM1	7.9	4.0	2.6	10.5	27.6	47.3	–	1.5	
ab-PCM2-II	–	–	–	5.6	13.1	81.2	11.7	2.5	

–, not detected; +, trace amount; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose.

mannose and galactose than those from the one with bran extract. The polysaccharides wc-PCM2 and wb-PCM2 from the wild strain of *Poria cocos* (No.P0) contained relatively more α-(1→3)-D-glucan and relatively higher molecular mass than those of No.5.78, as shown in Tables 1 and 2.

Table 2 shows the results from in vivo assay of the antitumor activities of the tested samples. No obvious antitumor activities were observed in ac-PCM0 and ac-PCM1, indicating that they were ineffective in suppressing the growth of the Sarcoma 180 tumor cells. Only ac-PCM2 had bioactivity, with an inhibition ratio of 42.3% and a P value less than 0.01. However, wc-PCM0, wc-PCM1 and wc-PCM2 exhibited significantly higher antitumor activities against the growth of Sarcoma 180 tumor cells, showing the inhibition ratios of 42.7% ($P \leq 0.05$), 56.0% ($P \leq 0.01$), and 66.8% ($P \leq 0.01$), respectively. It was noteworthy that wb-PCM and ab-PCM isolated from culture medium with bran extract did not have significant antitumor activities. The antitumor activities of the heteropolysaccharides from the mycelia cultivated with wild strain (No.P0) were higher than those from No.5.78 strain in the same medium containing corn steep liquor. Antitumor activity data for wb-PCM2 and ab-PCM2 were not determined due to an insufficient amount of these polysaccharides for in vivo experiments. The foregoing differences in antitumor activity among the various polysaccharides were probably due to their different molecular mass and monosaccharide composition, originating from the different strains and culture media. From the standpoint of structure, the antitumor activities of the polysaccharides

Table 2
Antitumor activities of heteropolysaccharides from *Poria cocos* mycelia against Sarcoma 180 solid tumor grown in BALB/c Mice

Sample	$[\eta]$ ($cm^3\ g^{-1}$)	$M_w \times 10^{-4}$ ($g\ mol^{-1}$)	Dose (mg/kg × days)	Inhibition ratio (%)	Complete repression
wc-PCM0	9.4	9.2	20 × 10	42.7 *	1/8
wc-PCM1	13.7	26.2	20 × 10	56.0 **	0/8
wc-PCM2	62.4	89.2	20 × 10	66.8 **	3/8
ac-PCM0	8.0	10.1	20 × 10	no	0/7
ac-PCM1	10.2	12.5	20 × 10	12.7	1/7
ac-PCM2	22.6	17.0	20 × 10	42.3 **	0/7
wb-PCM0	9.6	14.4	20 × 10	16.7	0/8
wb-PCM1	14.1	33.3	20 × 10	20.8	0/8
ab-PCM0	4.4	9.3	20 × 10	8.33	0/8
ab-PCM1	29.3	5.7	20 × 10	4.17	0/8

no, no inhibition of the growth of Sarcoma 180 solid tumor in BALB/c mice.
* $P < 0.05$.
** $P < 0.01$.

seemed to correlate positively with the amount of α-(1 → 3)-D-glucan bound with protein and the presence of mannose and galactose. In addition, relatively higher molecular mass and better water solubility seemed to increase the antitumor activities of the polysaccharides, as shown in the case of wc-PCM2 and ac-PCM2.

The inhibition ratios of tumor cell (suspended HL-60 leukemic cell and adherent $HepG_2$ cell) growth by the polysaccharides from *Poria cocos* mycelia with wc and ac strains are shown in Figs. 1 and 2. Regarding the antiproliferation of suspended HL-60 leukemic cells, the samples wc-PCM0, wc-PCM1, wc-PCM2, and ac-PCM0, ac-PCM1, ac-PCM2 all had relatively stronger inhibitions of tumor cell growth at concentrations of 200 and 100 μg/mL. However, the heteropolysaccharides wb-PCM0, wb-PCM1, ab-PCM0, and ab-PCM1 exhibited lower activities than corresponding polysaccharides cultivated in medium containing corn steep liquor.

In the MTT assay, the samples wc-PCM0, wc-PCM1, wc-PCM2 and ac-PCM0, ac-PCM1, ac-PCM2 showed only very low inhibition against the proliferation of adherent $HepG_2$ tumor cells, and in the case of wb-PCM0, wb-PCM1 and ab-PCM1 even proliferation of the cancer cells was observed. No antiproliferation effect of these polysaccharides on Vero cells was observed (data not shown). The foregoing results indicated that the heteropolysaccharides extracted from *Poria cocos* mycelia cultured in both media exhibited significant inhibition against the proliferation of suspended HL-60 tumor cells when compared to adherent $HepG_2$ tumor cell proliferation.

From these results, it may be concluded that the heteropolysaccharides from *Poria cocos* mycelia cultured with wild strain in a medium containing corn steep liquor exhibited the highest antitumor activities against Sarcoma 180 in vivo, and the heteropolysaccharides from mycelium cultured in media with bran extract did not show significant inhibition of tumor growth. However, the heteropolysaccharides used in this work all showed significant inhibition or cytotoxic effect against the suspended proliferation of HL-60 tumor cells in vitro. In general, polysaccharides isolated from the different strains of *Poria cocos* mycelia seemed to have different in vivo and in vitro antitumor activities, depending on their monosaccharide composition, protein content, molecular mass, and chain conformation.

1. Materials and methods

1.1. Preparation of polysaccharides

A strain of *Poria cocos* coded as No.P0 was obtained from wild *Poria cocos* in Luotian (Hubei, China), and another strain coded as No.5.78 was obtained from the Chinese Academy of Sciences (Beijing, China). The *Poria cocos* mycelia were cultured according to our previous work[18–20] and the products coded as wb-, wc-, ab- and ac-PCM. Accordingly, 'wb' and 'wc' mean No.P0 strain from wild (w) cultured in the medium containing bran extract (b) and corn steep liquor (c), respectively. Similarly, 'ab' and 'ac' mean No.5.78 strain from Chinese Academy of Sciences (a) cultured in medium b and c, respectively. *Poria cocos* mycelia were then isolated using 0.9% NaCl and hot water at 120 °C to obtain water-soluble heteropolysaccharides coded as PCM1 and PCM2.[18–20] The collected culture media were treated with MeOH to precipitate the water-soluble exo-polysaccharide coded as PCM0. Each polysaccharide was purified by procedures described else-

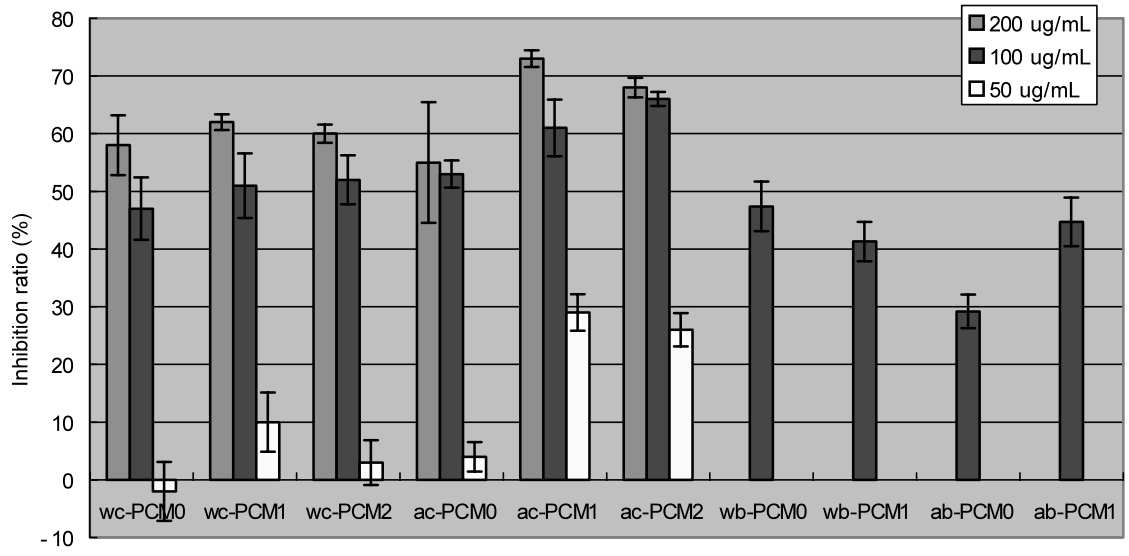

Fig. 1. Inhibition of proliferation of HL-60 leukemic cells by different concentrations of heteropolysaccharides from *Poria cocos* mycelia.

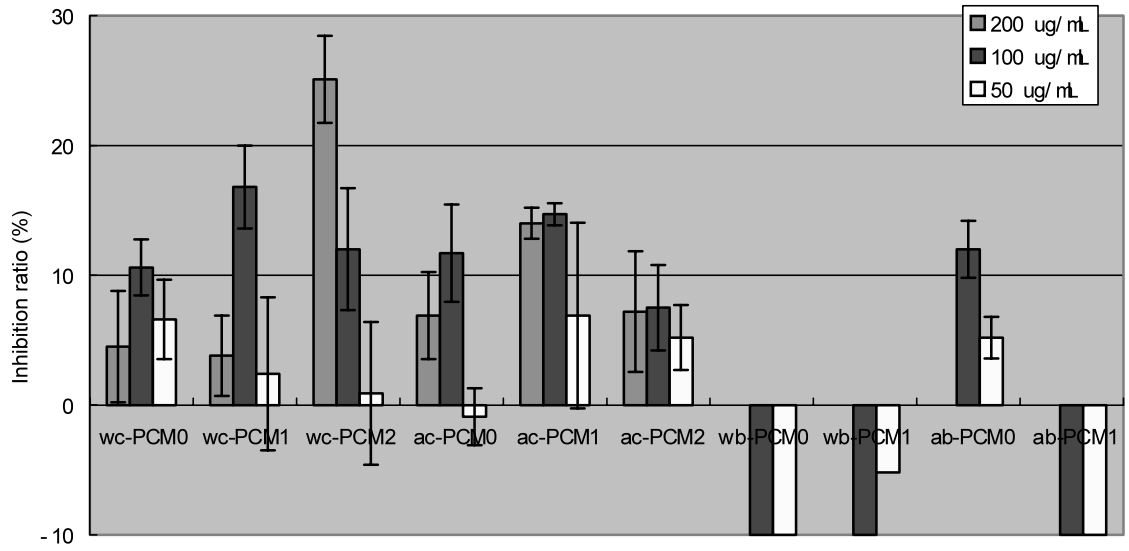

Fig. 2. Inhibition of proliferation of HepG2 liver cancer cells by different concentrations of heteropolysaccharides from *Poria cocos* mycelia.

where[21] and then lyophilized (Christ Alpha 1-2, Germany).

The chemical compositions and structural analysis of the polysaccharide fractions were determined by using a Nicolet FT-IR spectrometer (IR spectra), a Kjeletc 1030 semimicro Kjeldahl automatic analyzer (protein content), a Bruker DRX-400 NMR spectrometer (^{13}C NMR spectra), and an HP 6890 gas chromatograph (monosaccharide composition). The molecular mass M_w and intrinsic viscosity $[\eta]$ were determined by a multi-angle laser light scattering (LLS, Dawn®DSP, Wyatt Technology Co.), size exclusion chromatography (TSK-GEL G5000 and G3000 PWXL column; 7.8 mm × 300 mm) combined with laser light scattering (SEC-LLS) and viscometry. Detailed experimental procedures had been described previously.[18–20]

1.2. Cell culture and additional chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO). Human acute promyelocytic leukemia HL-60 (ATCC CCL-240), human hepatocellular carcinoma HepG2 (ATCC HB-8065), and monkey normal kidney Vero (AACT CCL-81) cell lines were purchased from the American Type Culture Collection (Rockville, MD).

1.3. In vivo antitumor test

Sarcoma 180 tumor cells (1×10^5 cells/mouse) were subcutaneously inoculated into 8-week-old BALB/c male mice. The samples PCM0, PCM1, and PCM2 were dissolved in phosphate buffer solution (PBS; 8.812 g NaCl, 0.201 g KCl, 0.204 KH_2PO_4 and 1.150 g Na_2HPO_4 were dissolved in 1 L of ultra pure water) and injected intraperitoneally (i.p.) once daily for 10 days at 24 h after tumor inoculation. The same volume of PBS was injected i.p. into the control mice. The tumor was allowed to grow on the mice for 7 days before it was removed from the animal and weighed. The antitumor activity of the tested samples was expressed as an inhibition ratio (percent) calculated as $[(A-B)/A] \times 100\%$, where A and B are the average tumor weight of the control and treated groups, respectively.

1.4. In vitro proliferation and cytotoxicity assays

1.4.1. Dye exclusion method for suspended cells. The HL-60 leukemic cells (1×10^5 cells/mL) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum under an atmosphere of 5% carbon dioxide at 37 °C for 72 h containing polysaccharides at concentrations of 50, 100, and 200 μg/mL in PBS solution. The survival rate of the mammalian cells was assayed by a hemacytometer by counting living cells that excluded the Trypan Blue dye.

1.4.2. Colorimetric MTT method for adherent cells. Mammalian HepG2 cells and Vero cells (1×10^5 cells/mL) were incubated separately with the polysaccharides at concentrations of 50, 100, and 200 μg/mL and allowed to grow under the same conditions as the HL-60 cells already mentioned. The number of living HepG2 cells and Vero cells at the end of the 72 h incubation period was determined by a colorimetric assay based on the tetrazolium salt MTT as described by Mosmann.[22] In these two assays, the tested samples were compared with control samples in the absence of the heteropolysaccharides. All in vitro results were expressed as the

ratio of inhibition of tumor cell proliferation calculated as $[(A-B)/A] \times 100\%$, where A and B are the average number of viable tumor cells of the control and samples, respectively.

1.4.3. Statistics. Statistical evaluations in all experiments were performed by a student's *t*-test. A *P* value of less than 0.05 was considered significant.

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References

1. Chihara, G. *Intern. J. Orient. Med.* **1992**, *17*, 57–77.
2. Ochiai, T.; Isono, K.; Suzuki, T.; Koide, Y.; Gunji, Y.; Nagata, M.; Ogawa, N. *Intern. J. Immunother.* **1992**, *8*, 161–169.
3. Adachi, K.; Nanba, H.; Kuroda, H. *Chem. Pharm. Bull.* **1987**, *35*, 262–270.
4. Suzuki, I.; Hashimoto, K.; Oikawa, S.; Sato, K.; Osawa, M.; Yadomae, T. *Chem. Pharm. Bull.* **1989**, *37*, 410–413.
5. Jong, S. C.; Birmingham, J. M.; Pai, S. H. *J. Immunol. Immunopharmacol.* **1991**, *11*, 15–122.
6. Mizuno, T. *Intern. J. Med. Mush.* **1999**, *1*, 9–29.
7. Chihara, G.; Hamuro, J.; Maeda, Y.; Arai, Y.; Fukuoka, F. *Nature* **1970**, *225*, 943–944.
8. Kulicke, W. M.; Lettau, A. I.; Thielking, H. *Carbohydr. Res.* **1997**, *297*, 135–143.
9. Ohno, N.; Miura, N. N.; Chiba, N.; Adachi, Y.; Yadomae, T. *Biol. Pharm. Bull.* **1995**, *18*, 1242–1247.
10. Kishida, E.; Sone, Y.; Misaki, A. *Carbohydr. Polym.* **1992**, *17*, 89–95.
11. Saito, H.; Yoshioka, Y.; Uehara, N.; Aketagawa, J.; Tanaka, S.; Shibata, Y. *Carbohydr. Res.* **1991**, *217*, 181–190.
12. Gomaa, K.; Kraus, J.; Franz, G.; Roper, H. *Carbohydr. Res.* **1991**, *217*, 153–161.
13. Demleitner, S.; Kraus, J.; Franz, G. *Carbohydr. Res.* **1992**, *226*, 239–246.
14. Mizuno, M.; Morimoto, M.; Minato, K.; Tsuchida, H. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 434–437.
15. Kiho, T.; Yoshida, I.; Nagai, K.; Ukai, S.; Hara, C. *Carbohydr. Res.* **1989**, *189*, 273–279.
16. Zhuang, C.; Mizuno, T.; Shimada, A.; Ito, H.; Suzuki, C.; Mayuzumi, Y.; Okamoto, H.; Ma, Y.; Li, J. *Biosci. Biotechnol. Biochem.* **1993**, *57*, 901–906.
17. Japan Synthetic Rubber Co. Ltd., *Jpn. Kokai Tokkyo Koho*, JP 58, 138, 392, (C1 C12P19/04) 17 August, 1983.
18. Jin, Y.; Zhang, L.; Chen, L.; Lin, Y.; Chen, Y.; Cheung, P.C.; Chen, L.G. *Carbohydr. Res.* **2003**, this issue, see following communication doi: 10.1016/S0008-6215(03)00197-6.
19. Zhang, L.; Chen, L.; Jin, Y.; Lin, Y.; Cheung, P. C.; Chen, L. *Chin. J. Polym. Sci.* **2003**, *21* (4), 21–28.
20. Lin, Y.; Zhang, L.; Jin, Y.; Chen, L.; Zhang, M.; Han, H.; Cheung, P. C.; Chen, L. G. *Acta Polymeric Sin.* **2003**, *1*, 97–103.
21. Zhang, L.; Yang, L.; Ding, Q.; Chen, X. *Carbohydr. Res.* **1995**, *270*, 1–10.
22. Mosmann, T. *J. Immunol. Methods* **1983**, *63*, 55–63.