Polymer Journal (2010) 42, 256–260

www.nature.com/pj

ORIGINAL ARTICLE

Preparation of bioactive water-soluble pachyman hydrolyzed from sclerotial polysaccharides of *Poria cocos* by hydrolase

Chao Bian[1,2,4], Nana Xie[1,2,4] and Fusheng Chen[1,2,3]

Water-soluble pachyman (WSP) was prepared by enzymic hydrolysis of pachyman extracted from *Poria cocos* sclerotium using a 0.5 mol l^{-1} NaOH solution at the optimized conditions of 55 °C, pH 4, 4% pachyman, 2 U ml^{-1} hydrolase, and a hydrolysis time of 6 h. Two WSP fractions, WSP-1 and WSP-2, were prepared using SephadexG-100 to separate WSP. Both fractions were of the β-(1→3)-D-dextran structure and were only composed of glucose with no nucleic acid and protein. The composition was analyzed by ultraviolet spectroscopy and infrared spectroscopy, Congo Red reaction analysis and monosaccharide composition analysis. The effects of WSP (M_w 1.75×10^5), WSP-1 (M_w 1.86×10^6) and WSP-2 (M_w 3.58×10^4) on the proliferation of S180 tumor cells *in vivo* and *in vitro* were studied. The inhibitory ratios of the three tested WSPs on the proliferation of S180 tumor cells at 0.5 μg ml^{-1} *in vitro* were found to be 4.0 ± 0.6, 2.2 ± 0.1 and 2.6 ± 0.1%, respectively, and the inhibitory ratio of carboxymethyl pachyman (CMP), which was used as a control, was −0.3 ± 0.4%. WSP, WSP-1 and WSP-2 also exhibited significant inhibition on S180 tumor cells *in vivo* at a dose level of 200 mg kg^{-1} day^{-1}, with their inhibition ratios found to be 43.94, 41.57 and 39.81%, respectively. Furthermore, the three tested WSPs had no obvious effect on the weight of mice, whereas the administration of CMP resulted in a decrease in mice weight at the tested concentration.
Polymer Journal (2010) **42,** 256–260; doi:10.1038/pj.2009.329; published online 6 January 2010

Keywords: β-(1→3)-D-dextran; bioactivity; pachyman; *Poria cocos*; water-soluble pachyman

INTRODUCTION

Sclerotia of *Poria cocos* has been used as a traditional herbal medicine for several centuries[1] in oriental countries such as China, Japan and Korea[2] because of its various bioactivities, such as sedative and diuretic.[3] In the past, insoluble pachyman, with a structure consisting of β-(1→3) linear chains with β-(1→6) branch chains[4,5] and accounting for approximately 90% of dry sclerotia of *Poria cocos*,[6–8] was typically discarded as a waste product of the traditional treatment method of the herb. Nowadays, to improve the utilization ratio of *Poria cocos* sclerotia, several methods have been adopted to modify pachyman.[9–12] In some cases, *Poria cocos* sclerotia is ground into powder and added to flour or rice powder directly to make cakes (http://www.cnncn.net/zlk/24/binglei75.htm). However, the universal method to treat pachyman is through chemical reactions, which can improve water solubility and bioactivities of pachyman by resecting the β-(1→6) branched chain, leaving only β-(1→3) linear dextran, or introducing hydrophilic groups into pachyman, or improving some other characteristics.[7,9,10,12–15] For example, carboxymethyl pachyman (CMP) with antitumor and inhibitory tumor cell growth bioactivities[12,16,17] was produced by heating pachyman with chloroacetic acid in alkaline solution.[9] Correspondingly, sulfated pachyman, which can inhibit the growth of tumor cells, was prepared by treating pachyman with chlorosulfuric acid in pyridine solution.[18–20] As a downside, reagent residues and environmental pollution might result from chemical methods during pachyman extraction and modification.[10,13–15] Therefore, some scientists tried to use enzymes to modify pachyma under enzymic moderate hydrolytic conditions and no environmental forces,[21] but, owing to the high price of enzymes, this method is expensive. Therefore, research is needed to optimize the hydrolysis conditions to obtain the best possible results using the least amount of costly enzymes.

In this work, a kind of hydrolase was selected and applied to hydrolyze pachyman to obtain WSP. The bioactivities of the WSPs were studied *in vivo* and *in vitro*. The results will be beneficial to the application of pachyman with bioactivity.

EXPERIMENTAL PROCEDURE

Materials

Pachyman (35.6 g) was extracted from 50 g of dry *Poria cocos* sclerotia in powder form and a 0.5 mol l^{-1} NaOH solution according to the previous

[1]State Key Laboratory of Agricultural Microbiology, Wuhan, PR China; [2]College of Food Science and Technology, Huazhong Agricultural University, Wuhan, PR China and [3]Key Laboratory of Food Safety Evaluation of the Ministry of Agriculture, Wuhan, PR China
[4]These authors contributed equally to this work.
Correspondence: Dr F Chen, College of Food Science and Technology, Huazhong Agricultural University, Wuhan, 430070, PR China.
E-mail: chenfs@mail.hzau.edu.cn
Received 30 July 2009; revised 28 September 2009; accepted 2 November 2009; published online 6 January 2010

method proposed by Zhang *et al.*[22] A sample of 10 g of pachyman was dissolved in 200 ml of NaOH (0.5 mol l^{-1}) and filtered, the pH of the filter liquor was adjusted to 7.0 using 36% acetic acid. The resulting pachyman emulsion was kept as a standby.

Carboxymethyl pachyman was synthesized according to the method published in 1996.[23]

The hydrolase adopted in this study was selected from a set of four different enzymes, Ultraflo XL (Novozymes, Copenhagen, Denmark), Ceremix 2XL (Novozymes), Viscozyme L (Novozymes) and Ultraflo Max (Novozymes), which we refer to as A, B, C and D, respectively. The WSP yield hydrolyzed by WSP produced from the reaction of each enzyme (0.5 U ml^{-1}) with 10 ml of pachyman emulsion under 50 °C, pH 7 for 2 h was measured. The yields of A, B, C and D were approximately 0.5, 2, 3.5 and 2%, respectively, from which C was selected for the preparation of WSP.

Sarcoma180 tumor cells from S180-bearing mice were bought from Huazhong University of Science and Technology. Six-week-old Kunming mice at a 50:50 male/female ratio, with a weight of approximately 20 ± 2 g, were brought from the Experimental Animal Center of Health and Epidemic Prevention Station.

Preparation of WSPs

The hydrolysis conditions used to obtain WSP from pachyman were optimized in a preparation experiment. Once factors such as temperature, pH, pachyman, hydrolase and hydrolysis time were optimized, 46 g of WSP were prepared from 80 g of pachyman, and two fractions, WSP-1 and WSP-2, were separated from WSP by SephadexG-100.

Analysis of WSPs

Ultraviolet spectra analysis of WSPs. WSP and its fractions were dissolved in distilled water at a concentration of 0.5 mg ml^{-1}. Absorption characteristics were measured within the wavelength range of 200 to 600 nm using distilled water as a reference substance.

Infrared analysis of WSPs. To prepare samples for infrared analysis, a small amount (1 mg) of WSP was ground with 100 mg of KBr and formed into tablets. Infrared spectroscopy was used to analyze the structure of polysaccharide.

Congo Red reaction of WSPs. NaOH (1 mol l^{-1}) was added to a 1:100 volume ratio mixture of Congo Red solution (50 μmol l^{-1}) and WSP (0.5 mg ml^{-1}) in amounts chosen to make solutions with terminal concentrations of NaOH of 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45 and 0.50 mol l^{-1}. Following the reaction under ambient temperature for 10 min, the solutions were scanned at wavelengths ranging between 400 and 600 nm. From the scans, the relationship between maximum absorption of Congo Red and the concentration of NaOH could be obtained. The control substances were prepared following the process described above, with the WSP replaced with distilled water.

Monosaccharide composition analysis. The three tested samples (5 mg) were boiled with H_2SO_4 (2 mol l^{-1}) for 4 h, centrifuged after neutralization by $BaCO_3$, and analyzed by thin layer chromatogram. Glucose, ribose, sucrose, galactose and fructose were prepared similarly and used as standards. The thin layer chromatogram was performed using a solution of butanol, isopropanol, acetic acid and water (in a volume ratio of 7:5:2:4 (V/V)) as a developer and aniline-diphenylamine as the chromogenic reagent. Results appeared after 15 min of chromogenic reaction at 100 °C. The R_f values of samples and standard panels were measured.

Measurement of weight-average molecular weights. The molecular weights (M_w) of WSP-1 and WSP-2 were detected by gel permeation chromatography. For the measurement, we used a PL aquagel-OH MIXED (300×7.5 mm, 8 μm) column eluted with water as the mobile phase at a flow rate of 1.0 ml min^{-1} of flow and a temperature of 35 °C with refractive index detector.

Antitumor activities of WSPs

Pretreatment of sarcoma 180 tumor cells. We pretreated the sarcoma 180 tumor cells before testing, according to the methods described below. S180 cells were obtained from ascites, which were extracted under aseptic situation from S180-bearing mice. The cells were washed twice in normal saline solution after centrifugation at 1000 r min^{-1} for 5 min. They were suspended in a solution consisting of 100 IU l^{-1} penicillin, 100 mg l^{-1} streptomycin and RPMI-1640 culture medium with 10% inactivated calf serum, for an overall concentration to 1×10^6 cells ml^{-1}. From this stock solution, 5 ml was shifted into a culture bottle and placed in an incubator for 24 h under a 5% CO_2 atmosphere, at 37 °C and saturated humidity.

Antitumor activities of WSPs in vitro. Activated S180 tumor cells in solution (with a concentration of 1×10^5 cells ml^{-1}, regulated by Roswell Park Memorial Institute (RPMI) 1640 medium) were inoculated on a 96-well cultivation plate, with 100 μl of solution per well. We then added WSP, WSP-1, WSP-2 or normal saline (25 μl) to the wells, in concentrations, to achieve terminal concentrations of WSP samples of 0, 0.05, 0.5, 5, 10, 50 and 100 μg ml^{-1}. The growth of cells was observed with an inverted microscope after a 24 h cultivation period under a 5% CO_2 atmosphere, at 37 °C and saturated humidity. Afterward, 20 μl of 2 mg ml^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution was added to each well, replacing the culture medium and allowing for a chromogenic reaction. The cultivation plate was kept in an incubated box for 4 h at 37 °C under 5% CO_2. Absorption at a wavelength of 570 nm was measured by an enzyme mark instrument after adding dimethyl sulfoxide (100 μl per well) instead of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and compared with control group that lacked WSPs. The growth inhibitor rate (ξ) of the tumor cells was calculated using the formula listed below:

$$\xi\ (\%)=\frac{A-B}{A}\times100$$

where A and B are the absorptions of the control group and experimental group, respectively. CMP was used as a reference for observing the growth inhibition rate of tumor cells.

Antitumor activities of WSPs in vivo. Kunming mice were inoculated with 0.1 ml of S180 tumor cells (1×10^7 cells ml^{-1}, 0.1 ml per mouse) that were obtained from a separate mouse that had undergone 7 days of immunization. The inoculated Kunming mice were weighed and assigned to 11 randomized groups (with 10 mice per group, 5 male and 5 female) including an experimental group, a positive control group and a negative control group. The experimental group was divided into three parts (with 3 groups per part), named WSP, WSP-1 and WSP-2, to which 50, 100 and 200 mg kg^{-1} day^{-1} (the weight of samples per mouse body weight ·day) were injected once a day for 7 days, and the positive and negative control groups were injected with 30 mg kg^{-1} day^{-1} cyclophosphamide and isovolumetric normal saline, respectively. After 7 days, the mice were weighed and the tumor mass was extracted from the mice and weighed. The tumor inhibitory rate (f) was calculated according to the equation below, and from this value the effects of WSP, WSP-1 and WSP-2 on the growth inhibition of S180 tumor cells could be explained.

$$f\ (\%)=\frac{M_c-M_e}{M_c}\times100$$

M_c and M_e refer to the average tumor weight of the control group and experimental group, respectively.

RESULTS

Preparation of WSPs

Temperature, pH, hydrolysis time, and the concentrations of pachyman and hydrolase were all optimized using C (β-glucanase, Novozymes) as catalyzer to hydrolyze pachyman. The optimal conditions were found to be as follows: 55 °C, pH 4, 4% pachyman, 2 U ml^{-1} hydrolase, and a hydrolysis time of 6 h. WSP (46 g) was obtained from pachyman (80 g) under the optimal conditions.

The two fractions (WSP-1 and WSP-2) were separated from WSP using a Sephadex G-100 (Figure 1). WSP-1 and WSP-2 were collected from the 6th to the 10th tube and from the 12th to the 17th tube, respectively. The collected fractions were concentrated

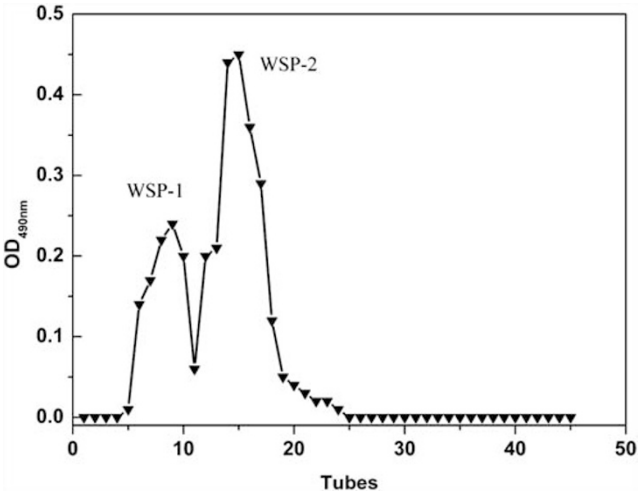

Figure 1 Elution curve of WSPs using a SephadexG-100 column. WSP-1 and WSP-2 were separated using a SephadexG-100. WSP-1 appeared earlier, in the 6th to the 10th tubes, whereas WSP-2 existed in the 12th to 17th tubes.

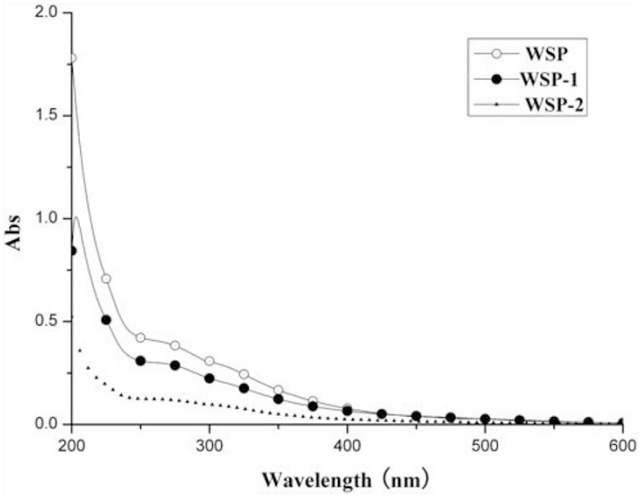

Figure 2 UV spectra of WSP, WSP-1 and WSP-2.

and used for structure analysis and measurements of the antitumor properties.

Analysis of WSPs

Ultraviolet spectrum analysis of WSPs. The ultraviolet (UV) spectra of WSP, WSP-1 and WSP-2 at 0.5 mg ml^{-1} are shown in Figure 2. All the samples exhibited the characteristic UV absorption attributed to polysaccharide with no visible absorption correlated with protein and nucleic acid at 280 and 260 nm, respectively. This means that the WSPs prepared in this study did not contain protein or nucleic acid.[22–25]

Infrared analysis of WSPs. There was no evident difference in the absorption of pachyman between WSP, WSP-1 and WSP-2 (Figures 3a–d). The infrared spectrum of three WSPs showed two peaks near 3800 and 3333 cm^{-1}, indicating that there is a stretching vibration of O-H. There were also peaks at 2914, 2916 and 2915 cm^{-1}, respectively, attributed to the stretching vibration of C-H, which is present in the pachyman spectrum. Moreover, features appearing at 2140, 2132 and 2138 cm^{-1} were 1134 frequency multiplication peaks, whereas the absorption between 1606 and 1650 cm^{-1} can be attributed to the bending vibration of O-H. The absorption peaks near 1420 and 1370 cm^{-1} are due to the deformation vibration of C-H. Peaks at 891 ± 7 cm^{-1} indicate the presence of compounds with a β-D-configuration,[15,26] and all of the three WSPs and pachyman own the configuration, which means that the β-D-configuration cannot be destroyed by hydrolase.

Congo Red reaction of WSPs. Congo Red reaction is used to determine the type and structure of the polysaccharides present by observing the complex formed between Congo Red and β-(1→3)-dextran. A red shift in the maximum absorption is induced when the concentration of NaOH lies between 0–0.5 mol l^{-1}. The reaction does not proceed between Congo Red and other polysaccharides or dextran without the β-(1→3) structure.[27]

The results of the Congo Red reaction are shown in Figure 4. The NaOH content in the samples ranges from 0.00 to 0.50 mol l^{-1}, and the maximum absorption of Congo Red mixed with the three WSPs moved toward the long wavelength side in all cases. A mixture of distilled water and Congo Red was used as a blank control. These results prove the existence of β-(1→3)-D-backbones, which could contribute to distinct antitumor activity.[28]

Monosaccharide composition analysis. The thin layer chromatogram method was adopted to analyze the monosaccharide content in the samples, according to the procedures introduced by Zhang *et al.*[29] A schematic of the process is shown in Figure 5. For WSP, WSP-1 and WSP-2, only a blue-gray spot was obtained after the chromogenic process, the R_f value of which was similar to that of the standard of glucose. This implied that glucose was the only component of WSPs.

Measurement of weight-average molecular weights. Molecular weight has a large effect on the water solubility as well as the bioactivities of polysaccharide. Using treated Dextrant (Molar mass 2×10^6, 5×10^5, 7×10^4, 4×10^4, 1×10^4) as a standard sample, the M_ws of WSP-1, WSP-2 and WSP (calculated on the basis of M_w of WSP-1 and WSP-2) were determined using gel permeation chromatography as 1.86×10^6, 3.58×10^4 and 1.75×10^5, respectively.

Antitumor activities of WSPs

Antitumor activities of WSPs in vitro. The results of the assays of three WSPs and the CMP against S180 tumor cells *in vitro* are summarized in Table 1. All of the samples exhibited the obvious effect of suppressing S180 tumor cell growth when the dose was above 0.05 μg ml^{-1}. In general, the antitumor activity was increased with an increase in the dose. The three WSPs showed a higher degree of tumor cell growth inhibition compared with the CMP samples at a dose of 0.5 μg ml^{-1}, with a very significant difference ($P<0.01$). When the dose was increased to 10 μg ml^{-1}, the WSP-1 antitumor activity was not more significant than the control, whereas WSP and WSP-2 displayed a higher inhibition rate than CMP. Interestingly, when the dose was increased to 100 μg ml^{-1}, except for WSP-2, WSP-1 and WSP once again showed more perfect inhibition effects than that of CMP, with a very significant difference ($P<0.01$).

Antitumor activities of WSPs in vivo. Table 2 shows the inhibition ratios of WSP, WSP-1 and WSP-2 against S180 tumor growth *in vivo*. Compared with the positive control group of 30 mg kg^{-1} day^{-1} cyclophosphamide and the negative control group of normal saline, the inhibition rates of WSP, WSP-1 and WSP-2 in the range of

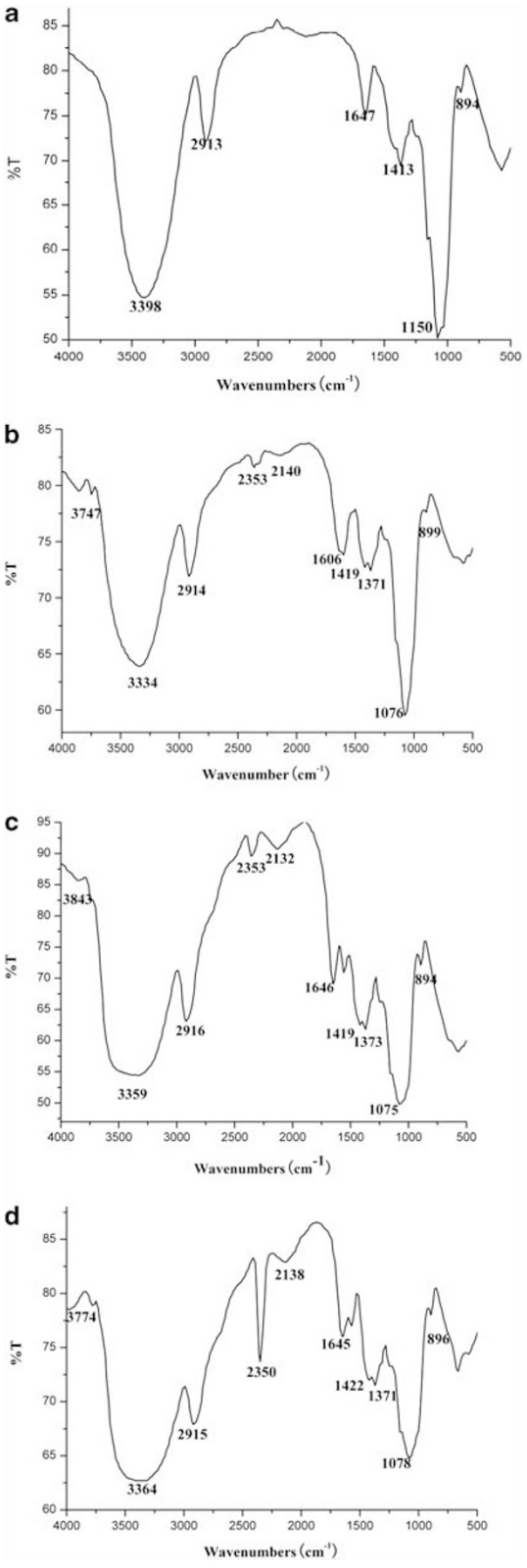

Figure 3 IR spectra of WSPs and pachyman. (**a**), (**b**), (**c**) and (**d**) are the IR spectra of pachyman, WSP, WSP-1 and WSP-2, respectively.

50–200 mg kg^{-1} day^{-1} were found to be 19.53–43.94, 20.32–41.57 and 11.45–39.81%, respectively. Although the tumor inhibitory effect of the positive control group was relatively high (60.21%), there was an

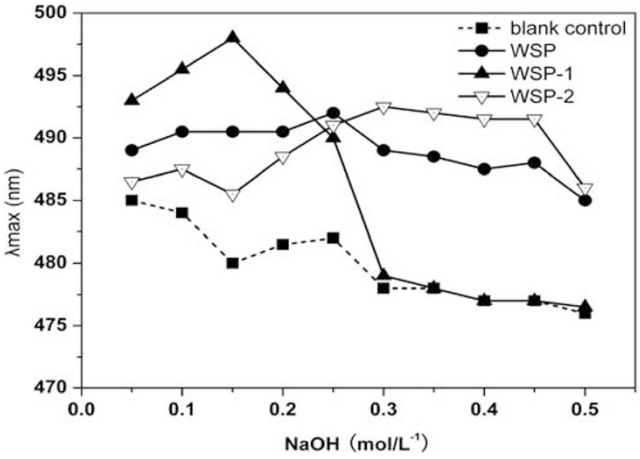

Figure 4 Effect of WSPs on the position of the maximum absorption of Congo Red.

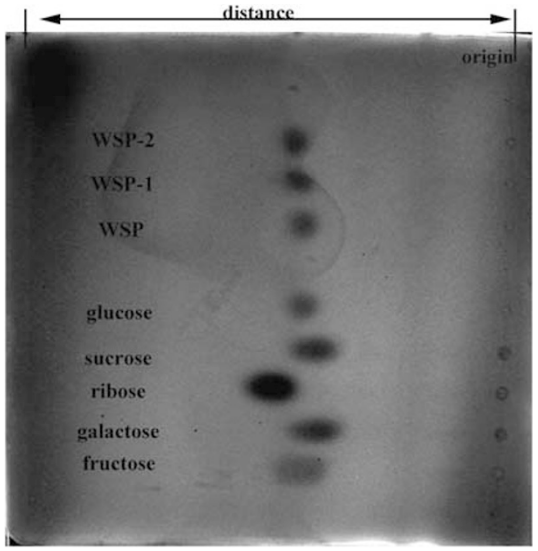

Figure 5 Thin layer chromatogram of WSPs for monosaccharide composition analysis. WSPs were analyzed by TLC using glucose, ribose, sucrose, galactose and fructose as standards. For WSP, WSP-1 and WSP-2, only a blue-gray spot was obtained after the chromogenic process, the R_f value of which was similar to that of the standard of glucose.

Table 1 Inhibition ratio of WSPs on S180 growth *in vitro* (%) ($\bar{x} \pm s$, $n=5$)

Concentration ($\mu g\,ml^{-1}$)	*CMP*	*WSP*	*WSP-1*	*WSP-2*
0.05	0.1 ± 0.1	0.0 ± 0.2	−1.0 ± 0.3	−1.1 ± 0.3
0.5	−0.3 ± 0.4	4.0 ± 0.6**	2.2 ± 0.1**	2.6 ± 0.1**
5	6.3 ± 0.5	4.7 ± 0.1**	4.2 ± 0.5**	3.3 ± 0.4**
10	8.1 ± 0.4	17.9 ± 0.2**	9.4 ± 0.6	9.9 ± 0.5**
50	19.1 ± 0.3	20.1 ± 0.4*	19.9 ± 0.5	19.0 ± 0.8
100	21.2 ± 0.7	26.2 ± 0.9**	26.6 ± 0.2**	20.4 ± 0.8

Abbreviations: CMP, carboxymethyl pachyman; WSP, water-soluble pachyman.
*$P<0.1$, significant difference. **$P<0.01$, very significant difference.

Table 2 Inhibition ratio of water-soluble pachymans (WSPs) on S180 tumor growth *in vivo* (%) ($\bar{x}\pm$ s, n=10)

Group	Dose ($mg\,kg^{-1}\,day^{-1}$)	Increase of body weight (g)	Tumor weight (g)	Tumor inhibitory rate (%)
Negative control	—	3.16 ± 1.42	1.23 ± 0.11	—
Cyclophosphamide	30	0.16 ± 0.05**	0.49 ± 0.07**	60.21
WSP	50	2.94 ± 0.81	0.99 ± 0.10	19.53
	100	2.61 ± 0.56	0.89 ± 0.12**	28.01
	200	3.22 ± 0.74	0.72 ± 0.08**	43.94
WSP-1	50	2.19 ± 0.69	0.98 ± 0.13	20.32
	100	2.38 ± 0.58	0.96 ± 0.06*	22.45
	200	2.31 ± 0.97	0.70 ± 0.07**	41.57
WSP-2	50	2.64 ± 0.21	1.09 ± 0.08	11.45
	100	2.36 ± 1.01	0.98 ± 0.10*	20.32
	200	2.27 ± 0.76	0.74 ± 0.04**	39.81

Negative control: normal saline.
*P<0.1, significant difference; **P<0.01, very significant difference.

evident decrease in the body weight of the mice, which was in contrast to effects observed for the negative control group. Body weight decline did not appear in the groups of mice treated with the WSPs. It is noteworthy that cyclophosphamide seems to affect the normal cells as well as tumor cells. By contrast, the three WSPs exhibited antitumor activities, arising from the stimulation of the immunoregulation mechanism of the host, which had little effect on normal cells.

DISCUSSION AND CONCLUSION

Pachyman extracted from *Poria cocos* sclerotium using 0.5 $mol\,l^{-1}$ NaOH solution was hydrolyzed under optimized conditions of 55 °C, pH 4, 4% pachyman, 2 $U\,ml^{-1}$ hydrolase and 6 h hydrolysis time to obtain WSP, the yield of which was 57.9%. Two fractions, WSP-1 and WSP-2, were separated from WSP using Sephadex G-100. The M_w values of WSP-1 and WSP-2, as well as WSP (calculated on the basis of M_w values of WSP-1 and WSP-2), were determined by gel permeation chromatography as 1.86×10^6, 3.58×10^4 and 1.75×10^5, respectively. The three WSP samples were composed of β-(1→3)-D-dextran with no nucleic acid or protein, evidenced from results of UV, infrared spectroscopy and Congo Red reaction and Monosaccharide composition analysis. In general, WSP, WSP-1 and WSP-2 showed obvious inhibition on S180 tumor cells *in vitro* at 10 and 100 $\mu g\,ml^{-1}$, outperforming the inhibition properties of CMP. The WSPs simultaneously had a perfect effect against S180 tumor cells at a dose level of 200 $mg\,kg^{-1}\,day^{-1}$ *in vivo,* attributed to the enhanced stimulation of the immunoregulation mechanism in the mice. WSPs that were hydrolyzed by β-glucanase had excellent antitumor activities on the growth of S180 as well as HeLa, CCL-220 and SP2 tumor cells. Details of these experiments will be given in a subsequent publication.

1 Chen, Y. Y. & Chang, H. M. Antiproliferative and differentiating effects of polysaccharide faction from Fu-ling (*Poria cocos*) on human leukemic U937 and HL-60 cells. *Food Chem. Toxicol.* **42,** 759–769 (2004).
2 Toshiyuki, A., Nobuko, K. & Masayuki, M. DNA Sequencing analysis of ITS and 28S rRNA of *Poria cocos*. *Biol. Pham. Bull.* **30,** 1472–1476 (2007).
3 Zhang, L. N., Chen, L., Xu, X. J., Zeng, F. B. & Cheung, P. C. K. Effect of molecular mass on antitumor activity of heteropolysaccharide from *Poria cocos*. *Biosci. Biotechnol. Biochem.* **69,** 631–534 (2005).
4 Saito, H., Misaki, A. & Harade, T. A. Comparison of the structure of cardlan and pachyman. *Agric. Biol. Chem.* **32,** 1261–1269 (1968).
5 Hoffman, G. C., Simson, B. W. & Timell, T. E. Structure and molecular size of pachyman. *Carbohydr. Res.* **20,** 185–188 (1971).
6 Cheung, P. C. K. Dietary fibre content and composition of some edible fungi determined by two methods of analysis. *J. Sci. Food Agric.* **73,** 255–260 (1997).
7 Li, Y., Wan, D. G., Liu, Z. G. & Yang, S. Studies on polysaccharide from the mycelium of fermented *Poria cocos* and natural *Poria cocos*. *Chem. Res. Application* **18,** 667–669, 673 (2006).
8 Chen, C. X., Zhao, D. M., Zhang, X. M. & Lin, Z. B. Anti-tumor experiment of carboxymethyl tuckahoe polysaccharide. *Fujian J Traditional Chinese Med* **33,** 38–40 (2002).
9 Wang, Y. F. & Zhang, L. N. Chain conformation of carboxymethylated derivatives of (1→3)–β-D-glucan from *Poria cocos* sclerotium. *Carbohydr. Polym.* **65,** 504–509 (2006).
10 Meng, Y. L., Cai, L. H., Wu, H. F. & Zhang, L. N. Immunohistochemical observation on the anti-tumor effect of the pachyman polysaccharides modified by chemical technique. *Wuhan Daxue Xuebao* **28,** 67–69 (2007).
11 Li, X. L., Jiao, Q. C., Chen, Q. & Liu, Q. Study on preventive and therapeutic effect of sulfated pachymaran in rats with chronic renal failure. *Chinese Pharm. J* **40,** 908–911 (2005).
12 Wang, Y. F., Zhang, L. N., Li, Y. Q., Hou, X. H. & Zeng, F. B. Correlation of structure to antitumor activities of five derivatives of β-glucan from *Poria cocos* sclerotium. *Carbohydr. Res.* **399,** 2567–2574 (2004).
13 Chihara, G., Hamuro, J., Maeda, Y. Y., Arai, Y. & Fukuoka, F. Antitumour polysaccharide derived chemically from natural glucan (pachyman). *Nature* **225,** 943–944 (1976).
14 Hamuro, J., Yamashita, Y., Ohsaka, Y., Maeda, Y. Y. & Chihara, G. Carboxymethylpachymaran, a new water soluble polysaccharide with marked antitumour activity. *Nature* **233,** 486–488 (1971).
15 Wang, Y. F., Zhang, M., Ruan, D., Shashkov, A. S., Kilcoyne, M., Savage, A. V. & Zhang, L. N. Chemical components and molecular mass of six polysaccharides isolated from the sclerotium of *Poria cocos*. *Carbohydr. Res.* **339,** 327–334 (2004).
16 Chen, C. X. The antitumor activity and immune effect of carboxymethylpachymaran (CMP). *Acta Edulis Fungi.* **8,** 39–44 (2001).
17 Hou, A. J., Yang, Z. Q., Huang, J. J. & Jiang, H. Carboxymethyl pachymaran up-regulates dendritic cell's function in hepatitis B virus transgenic mice *in vitro*. *Wuhan Daxue Xuebao Nat. Sci.* **12,** 372–378 (2007).
18 Vogl, H., Paper, D. H. & Fanz, G. Preparation of a sulfated linear (1→4)-β-D-galactan with variable degrees of sulfation. *Carbohydr. Polym.* **41,** 185–190 (2000).
19 Huang, Q. L., Zhang, L. N., Cheung, P. C. K. & Tan, X. Evaluation of sulfated α-glucans *Poria cocos* mycelia as potential antitumor agent. *Carbohydr. Polym.* **64,** 337–344 (2006).
20 Zhang, L. N., Chen, L., Xu, X. J., Lin, Y. L., Cheung, P. C. K. & Kennedy, J. F. Comparison on chain stiffness of a water-insoluble (1→3)-α-D-glucan isolated from *Poria cocos* mycelia and its sulfated derivative. *Carbohydr. Polym.* **59,** 257–263 (2005).
21 Chen, L. & Yu, J. P. Optimization of fuling (*Poria cocos*) polysaccharides extraction technology. *Food Sci.* **28,** 136–139 (2007).
22 Zhang, L., Ding, Q., Zhang, P. Y. & Feng, H. Q. Isolation and structural analysis of polysaccharides from the sclerotium of *Poria cocos* wolf. *Chem. J. Chinese Universities* **16,** 990–993 (1997).
23 Chen, C. X. The preparation and determination of carboxymethylpachymaran (CMP). *Acta Edulis Fungi* **3,** 31–36 (1996).
24 Ge, Q., Zhang, A. Q. & Sun, P. L. Research progress in structural modifications of polysaccharide in fungi and their identification. *Edible Fungi of China* **27,** 5–8 (2008).
25 Liu, H. & Zhang, J. Determination of molecular structure of carboxymethyl pumpkin polysaccharide by gas chromatography and IR spectroscopy. *Chinese J Spectroscopy Lab* **25,** 313–318 (2008).
26 Zhou, P., Xie, M. Y. & Fu, B. Q. A review of the studies on the polysacchride structure. *J Nanchang University* **25,** 197–204 (2001).
27 Ruan, Z., Hu, X. B., Chen, H., Wu, M. C. & De, Z. Y. Characterization of polysaccharide L-2A isolated from lentinus edodes. *Chinese J Spectroscopy Lab* **24,** 496–500 (2007).
28 Kojima, T., Tabata, K., Itoh, W. & Yanaki, T. Molecular weight dependence of the antitumor activity of Schizophyllan. *Agric. Biol. Chem.* **50,** 231–232 (1986).
29 Zhang, Z., Zou, Q. G., Song, Z. & Wu, R. J. Study of monosaccharides in PSK by TLC and HPLC-ELSD. *J China Pharm. University* **32,** 137–140 (2001).