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Carbohydrate Polymers 56 (2004) 123–128
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Molecular weight and anti-tumor activity of the water-soluble polysaccharides isolated by hot water and ultrasonic treatment from the sclerotia and mycelia of *Pleurotus tuber-regium*

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Received 9 July 2003; revised 19 December 2003; accepted 20 January 2004

Available online 12 March 2004

Abstract

The sclerotia (S) and mycelia (M) of *Pleurotus tuber regium* were extracted, respectively, by hot water (h) and ultrasonication (s) to yield four water-soluble polysaccharides coded as Sh, Mh, Ss and Ms. All water-soluble polysaccharides were mixture of two fractions that contained predominantly polysaccharides with glucose as the major sugar and galactose and mannose as the minor component. The water-soluble extracts from mycelia seemed to have higher protein content than the ones from sclerotia. The ultrasonicated water extracts (Ms and Ss) contained glycan–chitin complexes with higher weight-average molecular weight (M_w) than that by hot water. Interestingly, the polysaccharides Mh and Sh extracted with hot water, which contained the major fractions with M_w in the range of $40-80 \times 10^4$, exhibited stronger in vivo (Sarcoma 180 solid tumor implanted in BALB/c mice) and in vitro (HL-60 tumor cell culture) anti-tumor activities than that obtained by ultrasonication. In view of these results, the anti-tumor activities of the water-soluble polysaccharides isolated from both sclerotia and mycelia of *P. tuber-regium* depended on the method of isolation that affected the M_w profile and conponent of the extracts. The effects of moderate M_w and protein content of the polysaccharides on the improvement of the bioactivities could not be negligible.

Keywords: Mycelia; Sclerotia; Pleurotus tuber-regium; Anti-tumor activity; Water-soluble polysaccharides; Molecular mass; Size exclusion chromatography-laser light scattering

1. Introduction

Sclerotia of *Pleurotus tuber-regium*, a wild and edible, dry compact mass of fungal hyphae indigenous in Africa (Zoberi, 1973), have been successfully cultivated in China in recent years (Huang, Guo, & Huang, 1996). The sclerotia of *P. tuber-regium* contained over 90% dry weight of total dietary fiber of which 60% dry weight of its non-starch polysaccharide (NSP) component was β-glucan (Cheung & Lee, 1998). Structural analysis of the hot alkali-soluble NSP from this mushroom has revealed that they are consisted of a main chain of (1 → 3)-β-D-glucopyranosyl units with every third unit having a (1 → 6)-β-D-glucopyranosyl branch on average (Zhang, et al., 2001). Moreover, these sclerotial NSPs also exhibited both in vivo and in vitro anti-tumor activities (Zhang, Cheung, & Zhang, 2001), similar to other known mushroom polysaccharides such as lentinan and schizophyllan currently used in cancer therapy (Borchers, Stern, Hackman, Keen, & Gershwin, 1999). Recently, the mycelia of *P. tuber-regium* have also been successfully cultivated by submerged fermentation (Wu, Cheung, Wong, & Huang, 2003). It is well-known that water extracts from fruiting bodies and culture mycelia from higher Basidiomycota mushrooms often contain anti-tumor or immunostimulating polysaccharides (Reshetnikov, Wasser, & Tan, 2001). However, the chemical characteristics and anti-tumor activities of the water-soluble polysaccharides from the sclerotia and mycelia of *P. tuber-regium* have never been reported before. In this research, we attempted to investigate the chemical composition and molecular weight as well as the anti-tumor activity of the polysaccharides in the water extracts isolated from the mycelia and sclerotia of *P. tuber-regium*. Different extraction methods including hot

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doi:10.1016/j.carbpol.2004.01.005

water and ultrasonication were used to evaluate their effect on the structure and anti-tumor activity of the mushroom polysaccharides.

2. Experimental

2.1. Materials

Sclerotia of *P. tuber-regium* were cultivated by the Sanming Mycological Institute in Fujan of China and the mycelia were produced from submerged fermentation using a basal medium that contained fructose, peptone, yeast extract and minerals (Wu et al., 2003). Human acute promyelocytic leukemia HL-60 (ATCC no. CCL-240) and monkey normal kidney Vero (AACT no. CCL-81) cell lines were purchased from the American Type Culture Collection (Rockville, MD). Phosphate buffer solution (PBS) was prepared by dissolving 8.812 g NaCl, 0.201 g KCl, 0.204 KH_2PO_4 and 1.150 g Na_2HPO_4 in 1 l ultra-pure water.

2.2. Extraction of polysaccharides

100 g powder of sclerotia and 20 g powder of mycelia were defatted with diethyl ether and acetone for 4 h, respectively. The defatted mushroom samples were divided into two equal portions and suspended separately in 0.9% NaCl aqueous solution. They were then subjected to 100 °C treatment for 3 h and ultrasonication for 2 h (VCX600, SONICS and MATERIALS INC. USA), respectively. The above extraction mixtures were centrifuged, and the supernatants obtained were dialyzed against distilled water for 2 days and freeze-dried to produce the hot water extracts from mycelia and sclerotia coded as Mh and Sh, as well as ultrasonicated aqueous extracts from mycelia and sclerotia coded as Ms and Ss.

2.3. Component analysis

The protein content in water-soluble polysaccharides was measured by using an automatic analyzer (KJELETC 1030, Switzerland) according to semi-micro Kjeldahl method. The carbohydrate content of the samples was determined by the phenol-sulfuric acid method (Dubois, 1956). In brief, 1 ml of sample solution was vortex mixed with 1 ml of 5% phenol in water before adding 5 ml of concentrated sulfuric acid rapidly from a glass dispenser. After standing for 30 min at room temperature, the absorbance of the sample solution was measured at 490 nm against the blank (prepared by substituting distilled water for the sample solution). The amount of total carbohydrates was determined by reference to a standard curve made from glucose. Sugar composition of the extracts was determined by a gas chromatography (GC) method, in which the alditol acetate derivatives of the neutral sugars was measured (Englyst, Quigley, & Hudson, 1994). The GC condition used was: injector temperature, 275 °C; column temperature, 220 °C; detector temperature, 275 °C; carrier gas, helium; and flow rate, 8 $cm^3 min^{-1}$. Under these conditions, a GC (HP6890) fitted with a flame-ionization detector and a wide-bore capillary column (30 mm × 0.75 mm i.d. Supelco SP-2330) at 210 °C allow accurate determination of the individual sugars in the standard sugar mixture within 10 min. (data not shown). Standard sugar mixture and internal standard (allose) were used for calibration and determination of the detector response factor of individual sugars. The uronic acid content of the samples was measured by spectrophotometry according to the colorimetric method reported by Englyst et al. (1994).

2.4. Characterization

Size exclusion chromatography (SEC) combined with a multi-angle laser photometer (DAWN DSP, Wyatt Technology Co., USA, SEC-LLS) was performed in a system with a p100 pump (Thermo Separation Products, San Jose, Japan) equipped with two columns, PSW5000 and PSW3000 (TSK) and the interferometric refractometer at 25 °C. The carrier solution was PBS, and the samples were dissolved in PBS with stirring. The carrier and sample solutions were made dust free by passing through a 0.45 μm Millipore filter and degassed before use. The injection volume was 200 μl, and the flow rate was 1.0 ml min^{-1}. The calibration of the photometer was done with ultra-pure toluene, and the normalization of the refractive index (RI) detector was done with bovine albumin monomer (SIGMA A-1900). The specific RI increment (dn/dc) at 633 nm and 25 °C was determined using an interferometric refractometer (Optilab/903, Wyatt Technology, USA). The dn/dc value was averaged to be 0.136 ml/g and was assumed to be constant over the sample elution. Astra software was utilized for the data acquisition and analysis.

2.5. In vivo anti-tumor test

Sarcoma 180 cells (1×10^5 cells/mouse) were subcutaneously inoculated into 8-week-old BALB/c male mice. The samples (20 mg/kg) dissolved in PBS were injected intraperitoneally (i.p.) once daily for 10 days starting 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 another 7 days before it was removed from the animal and weighed. The anti-tumor 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 weights of the control and treated groups, respectively.

2.6. In vitro proliferation and cytotoxicity assays

Dye exclusion method for suspended cells: The HL-60 leukemia cells (1×10^6 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 the samples at concentrations of 50, 100, and 200 μg/ml in PBS. The survival rate of the mammalian cells was assayed by counting living cells that excluded the Trypan blue dye using a hemacytometer.

Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method for adherent cells: Mammalian HepG2 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 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 (1983). In the above two assays, the samples were compared with control samples in the absence of the Mh, Ms, Sh and Ss. 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 numbers of viable tumor cells of the control and samples, respectively. All tested samples were carried out in triplicates.

2.7. Statistics

Data in all the bioassays were statistically evaluated by Student's t-test and differences with a P value of less than 0.05 were considered significant.

3. Results

3.1. Chemical composition of the water-soluble extracts

The yield of the four extracts were 4, 3.5, 3.6 and 3.2% for the polysaccharides Mh, Ms, Sh and Ss, respectively. The protein and carbohydrate content as well as the sugar composition of the four polysaccharides are summarized in Table 1. The water-soluble polysaccharides isolated from mycelia had higher protein content (10.7% in Mh and 7.4% in Ms) but lower carbohydrate content (51% in Mh and 65% in Ms) than those polysaccharides isolated from the sclerotia of *P. tuber-regium*(protein content of 5.1% in Sh and 4.2% in Ss; carbohydrate content of 77% in Sh and 69% in Ss). Four kinds of monosaccharides including mannose, glucose, galactose and *N*-acetyl glucosamine were found in the four polysaccharides except for the absence of *N*-acetyl glucosamine in Sh. The polysaccharides extracted with hot water from mycelia and sclerotia (Mh and Sh) had higher glucose content than the ultrasonicated aqueous extracts (Sh and Ss), especially for Sh having the highest glucose content of 85.4% (Table 1). Only a small amount of uronic acids (0.27%) was found in Mh, with almost no uronic acids (less than 0.01%) being found in the other three samples Ms, Sh and Ss.

Table 1
The protein content, sugar composition and total carbohydrate content in samples Ms, Mh, Ss, Sh

Sample	Monosaccharide content in polysaccharides (%)					Total carbohydrate content (%)	Protein content (%)
	Man	Glc	Gal	GlcNAc	Uronic acid		
Mh	9.4	73.1	14.3	3.1	0.5	51	10.7
Ms	23.0	44.2	14.2	16.8	Trace	65	7.4
Sh	7.7	85.4	6.9	–	Trace	77	5.1
Ss	16.8	59.7	12.5	11.1	Trace	71	4.2

Not detectable. Trace <0.01%.

3.2. Molecular weight determination

The SEC chromatograms showing the column eluent of the samples Mh, Ms, Sh and Ss detected by laser light scattering (LLS) 90° detector [marked by (a)] and interferometric refractometer [marked by (b)] are presented in Fig. 1. For Mh, peak 1 detected by both LLS detector and refractometer indicated that its corresponding composition had a weight percentage of 82.1%, which was obtained using the division principle of SEC chromatogram. The peak 1 showed very large M_w and molecular size, ascribing to the strong intensity detected by LLS. Usually, the signals detected by LLS are correlated to M_w and molecular size of the sample. The weight percentage of peak 2 in Fig. 1 was calculated to be 17.9% for the Mh. The peak 2 was not detectable by LLS detector, suggesting that its molecular size was quite small. Accordingly, the M_w values of the two fractions (peaks 1 and 2) for samples of Ms, Sh, and Ss could be calculated from Fig. 1, and the results are summarized in Table 2.

3.3. In vivo anti-tumor activity

The anti-tumor activities of the samples Mh, Ms, Sh and Ss against Sarcoma 180 solid tumor grown in BALB/c mice at a dosage of 20 mg/kg for 10 days are shown in Table 2. The results showed that the polysaccharides extracted with hot water such as Mh and Sh had pronounced inhibition ratio of 65.4 ($P < 0.05$) and 55.3% ($P < 0.05$). However, although the Ms and Ss extracted with ultrasonic treatment showed around 30% inhibition ratio to the Sarcoma 180 tumor cell growth, no significant results ($P > 0.05$) were observed.

3.4. In vitro anti-tumor activity

The inhibition ratio to the proliferation of HL-60 leukemic cells by different concentrations (200, 100 and 50 μg/ml) of the samples Mh, Ms, Sh and Ss is shown in

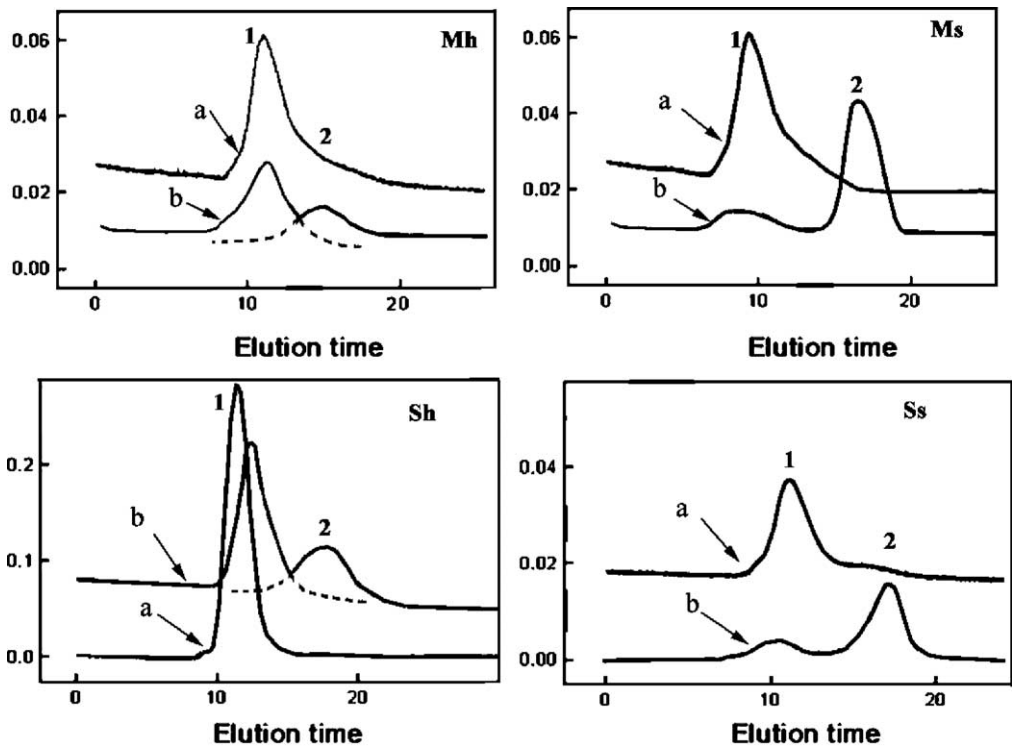

Fig. 1. SEC and LLS Chromatogram of polysaccharides Mh, Ms, Sh and Ss in PBS at 25 °C detected by laser light scattering photometry (a: LLS) and interferometric refractometer (b). The LLS data represents the 90° light scattering angle (detector 11).

Fig. 2. Basically, the four samples exhibited inhibition effect on HL-60 leukemia cell growth at three concentration levels tested. The four samples also showed a dose-response relationship in inhibiting the HL-60 cells proliferation. The Mh exhibited the highest inhibition ratio of 83 ($P < 0.05$), 69.9 ($P < 0.05$) and 56% ($P < 0.05$) to the proliferation of HL-60 cell line at the concentrations of 200, 100 and 50 μg/ml, respectively. Moreover, the Mh and Ms exhibited relatively higher inhibition ratio than Sh and Ss at the three concentrations, except for Ms at the concentration of 50 μg/ml. It was noted that the samples Mh and Sh isolated by hot water from mycelia and sclerotia had higher in vitro inhibition ratio than the ultrasonicated aqueous extracts Ms and Ss in corresponding concentrations. In addition, no anti-proliferation effect of the four samples on the normal Vero cells was observed (data not shown), suggesting that the cytotoxic effect of the Mh, Ms, Sh and Ss was preferential against tumor cells.

4. Discussion

A mixture of at least two polysaccharides with different chemical composition and M_w was obtained by extracting with both hot water and ultrasonic treatment from the sclerotia and mycelia of *P. tuber-regium*. The hot water treatment could yield polysaccharides with more glucose and less *N*-acetyl glucosamine and mannose as well as lower M_w than those obtained by ultrasonic method. This could be probably due to the more specific action of high energy sonication to degrade the fungal cell wall polysaccharide, releasing the glucan-chitin and mannan type of

Table 2
M_w and anti-tumor activities of polysaccharides Mh, Ms, Sh and Ss from sclerotia and mycelia of *P. tuber-regium*

Samples	Fraction 1		Fraction 2		Inhibition ratio (%)[a]	Complete regression
	$M_w \times 10^4$	Weight percentage (%)	$M_w \times 10^4$	Weight percentage (%)		
Mh	76.2	82.1	3.23	17.9	65.4*	0/15
Ms	186	7.91	4.27	92.1	30.8	0/15
Sh	43.5	73.2	2.87	26.8	55.3*	0/15
Ss	157	23.7	4.01	76.3	22.1	0/15

* $P < 0.05$, significant difference when compared to the control.
[a] In vivo anti-tumor activities against Sarcoma 180 solid tumor growth in BALB/c mice at a dosage of 20 mg/kg for 10 days.

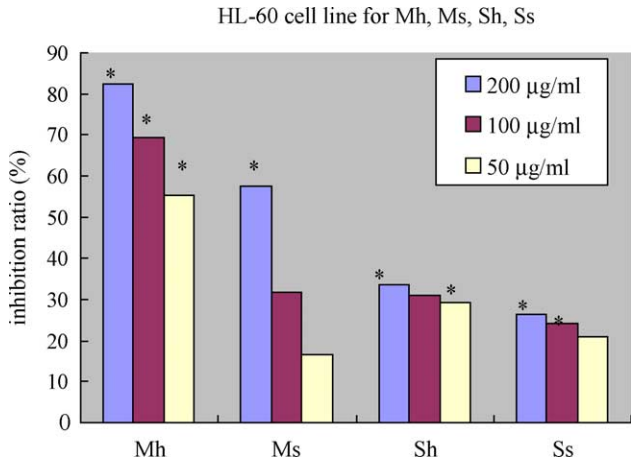

Fig. 2. Inhibition ratio (%) to proliferation of HL-60 leukemic cells by different concentrations (200, 100 and 50 μg/ml) of polysaccharides Mh, Ms, Sh and Ss. *indicates significant difference ($p < 0.05$) in the inhibition ratio of the samples compared with control at the same concentration.

polysaccharides with high M_w and polysaccharide fragments with low M_w into the aqueous medium (Mislovicova, Masarova, Bendzalova, Soltes, & Machova, 2000). Moreover, water-soluble extracts from sclerotia had lower protein content than those extracted from the mycelia, disregard the extraction methods. This was consistent with our previous result showing that mushroom sclerotia were known for its low protein content (Cheung & Lee, 1998).

NSP are important candidate macromolecules as the biological response modifiers implicated in cancer immunotherapy (Borchers et al., 1999). In order to explore and broaden the application of plant polysaccharides as much as possible in the field of food and medicine, a variety of mushroom and fungi has been studied for their polysaccharides as novel and potential anti-tumor agents (Reshetnikov et al., 2001). In our previous work, fractions of β-glucans extracted by hot alkali from the sclerotia of *P. tuber-regium* were demonstrated to have anti-tumor activity (Zhang et al., 2001). The polysaccharides isolated from the mycelia and scleortia of another edible mushroom such as *Poria cocos* have shown to have host-mediated, anti-tumor activities (Jin, Zhang, & Zhang, 2003a; Jin, Zhang, Lin, Chen, & Zhang, 2003b). In this research, the water-soluble extracts, which were rich in polysaccharides isolated from both the mycelia and the sclerotia, contained two fractions with different molecular weights. The fractions having M_w of 76.2×10^4, 4.27×10^4, 43.5×10^4 and 4.01×10^4 were considered as the main component in the polysaccharides Mh, Ms, Sh and Ss because of their higher weight percentage of 82.1, 92.1, 73.2 and 76.3%, respectively (Table 2). The Ms and Ss showed relatively lower in vivo and in vitro inhibition ratio to the tumor cell growth than those of the Mh and Sh (Table 2 and Fig. 2), indicating that extracts produced by the ultrasonication method contained the fractions having much higher and lower M_w (186×10^4 and 4.27×10^4 for Ms, 157×10^4 and 4.01×10^4 for Ss), which were less effective in inhibiting the tumor cell growth. Interestingly, hot water extraction seemed to be a better method to obtain anti-tumor polysaccharides from the mycelia and sclerotia of *P. tuber-regium*. The predominant fraction in the polysaccharides Mh and Sh had moderate molecular mass of 76.2×10^4 and 43.5×10^4. Moreover, the water-soluble polysaccharides Mh and Sh exhibited substantial anti-tumor activity. The results were similar to that of native Lentinan and schizophyllan ($M_w > 4 \times 10^5$) (Sasaki & Takasuka 1976; Yanaka, Norisuye, & Fujita, 1980). It was also found in our previous work that M_w is very important to the bioactivity of the mushroom polysaccharides. For example, an alkali-soluble native glucan isolated from the sclerotia of *P. tuber-regium* showed relatively higher anti-tumor activity in the M_w range from 10 to 20×10^4 (Zhang et al., 2001), while the fractions with higher or lower M_w were not effective in inhibiting the tumor cell growth both in vivo and in vitro. Moreover, their water-soluble carboxymethylated derivatives had higher inhibition ratio in a broader M_w range from 10 to 50×10^4 (unpublished data). In this work, the water-soluble polysaccharides with their major carbohydrate components having M_w in the range of 40 to 80×10^4 were demonstrated to have higher anti-tumor activity than those with major components having M_w below 5×10^4.

The bioactivity assay showed that the Mh and Sh extracted by hot water exhibited significantly both anti-tumor activity to solid tumor sarcoma 180 cells in vivo and direct cytotoxity to HL-60 tumor cell lines in vitro. These results were quite different from the other known anti-tumor native mushroom polysaccharides such as schizophyllan and lentinan (Fujii, 1978; Tabata, Ito, Kojima, Kawabata, & Misaki, 1981), all of which have no direct cytotoxicity to tumor cell lines in vitro. However, the water-soluble extracts which contained possibly proteoglycans were quite similar to those of protein-bound polysaccharide (PSK, Krestin), polysaccharopeptide (PSP) isolated from *Coriolus versicolor* that had both immunomodulating activities and direct cytotoxicity to a wide range of tumor cell lines including HL-60 (Ooi & Liu 2000; Sakagami, Aoki, Simpson, & Tanuma, 1991; Yang et al., 1992).

In view of the results mentioned above, the effects of protein content and moderate molecular weight (such as $1 \times 10^6 > M_w > 4 \times 10^5$) on the improvement of the bioactivities of the polysaccharides could not be negligible. Further structural analysis and evaluation of bioactivities to the polysaccharides from the mycelia, sclerotia and fruiting body of *P. tuber-regium* will be very important for their application in food and medicinal fields.

5. Conclusion

Four polysaccharides Mh, Sh, Ms and Ss were successfully isolated by extracting with hot water and ultrasonic

treatment from the mycelia and sclerotia of *Pleurotus tuber-regium*. The water-soluble extracts from sclerotia had lower protein content than those extracted from mycelia, disregard of the extraction method. The hot water treatment could yield polysaccharides Mh and Sh with more glucose and less *N*-acetyl glucosamine and mannose as well as lower M_w than those obtained by ultrasonic method. Hot water treatment is a better method than ultrasonic one to obtain polysaccharides,which have higher antitumor activities that is host-mediated and cytocidal, than those polysaccharides extracted by sonication method.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (20074025), the Earmarked Research Grants Council (CUHK 4255/02M) and the Area of Excellence (AoE) on Plant and Fungal Biotechnology project of the Hong Kong SAR Government. The authors thank the Sanming Mycological Institute in Fujian, China, for supplying the mycelia and sclerotia of *P. tuber-regium*.

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