BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Isolation and characterization of polysaccharides with the antitumor activity from *Tuber* fruiting bodies and fermentation system

Wei Zhao · Xiao-Hua Wang · Hong-Mei Li · Shi-Hua Wang · Tao Chen · Zhan-Peng Yuan · Ya-Jie Tang

Abstract Fifty-two polysaccharides were isolated from the fermentation systems of *Tuber melanosporum*, *Tuber indicum*, *Tuber sinense*, *Tuber aestivum* and the fruiting bodies of *Tuber indicum*, *Tuber himalayense*, *Tuber sinense* by elution with an activated carbon column. Polysaccharides from *Tuber* fermentation system exhibited relatively higher in vitro antitumor activity against HepG2, A549, HCT-116, SK-BR-3, and HL-60 cells than those from *Tuber* fruiting bodies. All polysaccharides were mainly composed of D-mannose, D-glucose, and D-galactose, which suggested that the polysaccharides from *Tuber* fruiting bodies and fermentation system have identical chemical compositions. The results of antitumor activity and structural identification indicated that the polysaccharide fractions could promote antitumor activity. *Tuber* polysaccharides from *Tuber* fermentation system exhibited relatively higher than that from *Tuber* fruiting bodies. These results confirm the potential of *Tuber* fermentation mycelia for use as an alternative resource for its fruiting bodies.

Keywords *Tuber* · Fermentation mycelia · Fruiting bodies · Polysaccharide · Antitumor activity

Wei Zhao and Xiao-Hua Wang equally contributed to this work

W. Zhao · H.-M. Li · Y.-J. Tang (✉)
Key Laboratory of Fermentation Engineering (Ministry of Education), Hubei University of Technology, Wuhan 430068, China
e-mail: yajietang@hotmail.com

X.-H. Wang · S.-H. Wang
Key Laboratory of Biopesticide and Chemical Biology (Ministry of Education), Fujian Agriculture and Forestry University, Fuzhou 350002, China

T. Chen
Key Laboratory of Systems Bioengineering (Ministry of Education), School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China

Z.-P. Yuan
School of Public Health, Wuhan University, Wuhan 430071, China

Introduction

Truffle, also known as "underground gold" is a popular ingredient in international cuisine because of its unique aroma and bioactive metabolites. Because of the high commercial demand for truffle and the shortage in wild resources, an improved means of producing truffles on a large scale would have important economic impact. As it usually takes 4–12 years to cultivate the fruiting body of truffle, a time-consuming process with unsteady quality, submerged culture is viewed as a promising alternative for the efficient production of bioactive metabolites (Zhong and Tang 2004). Our laboratory has successfully developed a truffle fermentation system, and systematically optimized the medium components, including the carbon source (Tang et al. 2008a, b), nitrogen source (Liu et al. 2008), metal ion (Tang et al. 2008a, b), and plant oil (Liu and Tang 2010). Based on these previous works, we qualitatively and quantitatively assayed the nucleosides and nucleobases in *Tuber* fruiting bodies and fermentation mycelia for the first time by developing an assay method that couples DSPE with LC-MS (Liu et al. 2011). A comparison of the lipid content and fatty acid composition of *Tuber* fermentation mycelia and natural fruiting bodies indicates that the lipid content in *Tuber* fermentation mycelia is

higher than that in fruiting bodies (Tang et al., 2011). In addition, a novel high-cell density fed-batch fermentation process was developed to produce *Tuber* polysaccharides (Liu et al. 2009), one of the fundamental nutrient substances. This method resulted in the highest ever reported yields of extracellular polysaccharides (EPS) and intracellular polysaccharides (IPS) at 7.09 ± 0.62 and 4.43 ± 0.21 g/L, respectively (Liu et al. 2009).

It is well known that polysaccharides are polymeric carbohydrate structures, formed of repeating units (either mono- or di-saccharides) joined together by glycosidic bonds. These structures are often linear, but may contain various degrees of branching. Polysaccharides are often quite heterogeneous, containing slight modifications of the repeating unit. Depending on the structure, these macromolecules can have distinct properties from their monosaccharide building blocks. They may be amorphous or even insoluble in water. Polysaccharides from certain mushrooms, including *Lentinus edodes*, *Ganoderma lucidum*, and *Pleurotus tuber-regium*, exhibit antitumor activity, and their physicochemical properties have been previously characterized (Zhang et al. 2007). Moreover, the water-soluble polysaccharide fractions from *P. tuber-regium* have been shown to inhibit HepG2 cells growth (Tao et al. 2006). Similarly, polysaccharides from *Poria cocos* fermentation mycelia and fruiting bodies of *Pleurotus ostreatus* can inhibit the tumor cell growth of HL-60 and HeLa, respectively (Huang et al. 2007; Tong et al. 2009). Furthermore, polysaccharides isolated from the fruiting body of *T. sinense* exhibited immune-modulating and antitumor activities (Hu et al. 1994). Despite the potential medical applications suggested by these previous studies, the bioactivities, structures, and physicochemical properties of the polysaccharides from *Tuber* fermentation system and fruiting bodies have not been thoroughly investigated, which prompted our efforts in this current study.

By isolating polysaccharide fractions from three *Tuber* fruiting bodies and four fermentation systems, we systematically assayed the fermentation environment affecting the molecular structure of polysaccharides and further affecting their biological activity for the first time. The central goal of this study was to investigate *Tuber* polysaccharides for in vitro antitumor activity, to determine the primary structure, and to understand the representative fractions structure–function relationship between *Tuber* fruiting bodies and fermentation mycelia.

Materials and methods

Truffle fruiting body collection and mycelia culture

T. indicum, *T. himalayense*, and *T. sinense* were all obtained from the middle reach area of Nujiang River (Yunnan, China),

and all fruiting bodies were stored at −20 °C. Truffle mycelia were cultured in our laboratory. The strains of *T. melanosporum*, *T. sinense*, and *T. indicum* were purchased from Mianyang Institute of Edible Fungi (Sichuan, China). The strain of *T. aestivum* was kindly provided by Huazhong Agricultural University (Hubei, China). The strain of *T. melanosporum* was cultured from both batch fermentation and fed-batch fermentation (Liu et al. 2008), while the other strains were all cultured only from batch fermentation (Tang et al. 2008a, b).

Sample preparation

Preparation of polysaccharides from Tuber fermentation mycelium by traditional methods

The polysaccharides of *T. melanosporum* fed-batch fermentation mycelium and batch fermentation broth were isolated and purified. Powder of the mycelium (100 g) was twice defatted with ethanol (75 °C, 3 h), extracted three times with water (100 °C, 3 h), and filtered to obtain the filtrate. The residue was divided into two parts extracted with 1 M NaOH and 1 M HCl aqueous solutions, respectively (25 °C, 8 h), and the filtrates were isolated. All filtrates and the batch fermentation broth of *T. melanosporum* were precipitated by adding 3BV ethanol (4 °C, 24 h), followed by centrifugation at 11,000 rpm for 30 min. Precipitates were filtered and dried to obtain crude polysaccharides. All of the crude polysaccharides were named as the form of "strain-synthetic route-extracted method". For example, "*T. i*" is the abbreviation of strain *Tuber indicum*. "BPS" represents the crude polysaccharide produced from the fruiting body. "1" represents the crude polysaccharides "*T. i*-BPS-1" was purified and separated with H_2O by activated carbon column method. The crude polysaccharides were then treated ten times to remove proteins by Sevag's procedure (Staub 1965), and freeze-dried for 5 days to obtain purified white cotton-like polysaccharides *Tuber melanosporum* batch fermentation mycelium polysaccharides-a (*T. m*-MPS-a), *T. melanosporum* batch fermentation mycelium polysaccharides-b (*T. m*-MPS-b), *T. melanosporum* batch fermentation mycelium polysaccharides-c (*T. m*-MPS-c), and *T. melanosporum* batch fermentation broth polysaccharides-a (*T. m*-FPS-a), respectively (Table 1). *T. m*-MPS-a, b, and c was extracted with H_2O, 1 M NaOH, and 1 M HCl by Sevag's method, respectively.

Preparation of Tuber polysaccharides by activated carbon method

Polysaccharides *Tuber indicum* fruiting body polysaccharides (*T. i*-BPS), *Tuber himalayense* fruiting body polysaccharides (*T. h*-BPS), *Tuber sinense* fruiting body polysaccharides

Table 1 Polysaccharides from different truffle resource and preparation method

Truffle resource		Purified and separated by activated carbon column				Purified by Sevag method			No extraction process
		Eluent				Extraction reagent			
		H$_2$O	0.9 % NaCl	1 M NaOH	1 M HCl	H$_2$O	1 M NaOH	1 M HCl	
BPS	T. i-BPS	T. i-BPS-1	T. i-BPS-2	T. i-BPS-3	T. i-BPS-4				
	T. s-BPS	T. s-BPS-1	T. s-BPS-2	T. s-BPS-3	T. s-BPS-4				
	T. h-BPS	T. h-BPS-1	T. h-BPS-2	T. h-BPS-3	T. h-BPS-4				
MPS	T. m-MPS	T.m-MPS-1	T.m-MPS-2	T.m-MPS-3	T.m-MPS-4				
	F-T. m-MPS	F-T. m-MPS-1	F-T. m-MPS-2	F-T. m-MPS-3	F-T. m-MPS-4	F-T. m-MPS-a	F-T. m-MPS-b	F-T. m-MPS-c	
	T. i-MPS	T. i-MPS-1	T. i-MPS-2	T. i-MPS-3	T. i-MPS-4				
	T. s-MPS	T. s-MPS-1	T. s-MPS-2	T. s-MPS-3	T. s-MPS-4				
	T. a-MPS	T. a-MPS-1	T. a-MPS-2	T. a-MPS-3	T. a-MPS-4				
FPS	T. m-FPS	T. m-FPS-1	T. m-FPS-2	T. m-FPS-3	T. m-FPS-4				T. m-FPS-a
	T. i-FPS	T. i-FPS-1	T. i-FPS-2	T. i-FPS-3	T. i-FPS-4				
	T. s-FPS	T. s-FPS-1	T. s-FPS-2	T. s-FPS-3	T. s-FPS-4				
	T. a-FPS	T. a-FPS-1	T. a-FPS-2	T. a-FPS-3	T. a-FPS-4				

(T. s-BPS) were prepared from the dried truffle fruiting bodies (T. indicum, T. himalayense, and T. sinense). Polysaccharides T. melanosporum batch fermentation broth polysaccharides (T. m-FPS), T. indicum fermentation broth polysaccharides (T. i-FPS), Tuber aestivum fermentation broth polysaccharides (T. a-FPS) were prepared from the batch fermentation broth. Polysaccharides T. melanosporum batch fermentation mycelium polysaccharides (T. m-MPS), T. melanosporum fed-batch fermentation mycelium polysaccharides (F-T. m-MPS), T. indicum fermentation mycelium polysaccharides (T. i-MPS), T. aestivum fermentation mycelium polysaccharides (T. a-MPS), T. sinense fermentation mycelium polysaccharides (T. s-MPS) were prepared from the fed-batch fermentation mycelium (Table 1). Each residue was further extracted three times with boiling water (3 h) and filtered. The combined aqueous extract of each residue was precipitated by adding 3BV ethanol (4 °C, 24 h) followed by centrifugation at 11,000 rpm for 30 min, filtered, and the precipitates dried to obtain crude polysaccharide (xPS). As shown in Fig. 1, the crude polysaccharide (0.5 g) was dissolved in 50 mL of water, and then purified and fractionated by activated carbon method. To confirm purity and polysaccharide properties, all fractions eluted from the activated carbon column were qualified by the anthrone–sulfuric acid method (Scott and Melvin, 1953), followed by protein assay using Coomassie brilliant blue. The batch fermentation broths of T. sinense, T. indicum, T. aestivum, and T. melanosporum were precipitated by adding 3BV ethanol (4 °C, 24 h), and then purified and fractionated by the activated carbon method. Except for the samples eluted with water, the resulting fractions were all dialyzed with regenerated cellulose films (MWCO 3500; BIOSHARP, USA).

Cell growth inhibition assay

The inhibitory effects of all polysaccharides on the growth of the human hepatocellular liver carcinoma cell line (HepG2 cells), human lung adenocarcinoma epithelial cell line (A549 cells), human colorectal carcinoma cell line (HCT-116 cells), human breast adenocarcinoma (SK-BR-3 cells), and the human promyelocytic leukemia cell line (HL-60 cells) cells were evaluated in vitro by micro culture tetrazolium-3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphen-yltetrazolium bromide (MTT; Sigma, St. Louis, MO) assay to measure cytotoxic effects. Drug stock solutions were prepared in dimethyl sulfoxide (DMSO). The final solvent concentration was ≤2 % (v/v) DMSO, which was sufficiently low to affect cell replication. Initial seeding densities were varied among the cell lines to ensure a final absorbance reading in the control (i.e., untreated) cultures in the range of 0.6–0.8 A_{492} units.

Inhibition of proliferation of adherent cells

HepG2, A549, HCT-116, and SK-BR-3 (3.5×10^4 cells/mL) were incubated in 96-well plates containing 100 μL of the culture medium at 37 °C in a humidified atmosphere with 5 % CO_2. Cells were permitted to adhere for 24 h, and then, each of 52 polysaccharides was added to each well at two final concentrations (20 and 200 μg/mL) in the culture medium. The negative control group (0.5 % DMSO) was established. After 72 h of incubation at 37 °C, the cells in each well were then incubated in culture medium with 20 μL of 5 mg/mL MTT solution for 3 h at 37 °C. After the medium was removed, 120 μL of DMSO were added to each well. Absorbance at 595 nm was determined by a Power Wave X Micro-plate
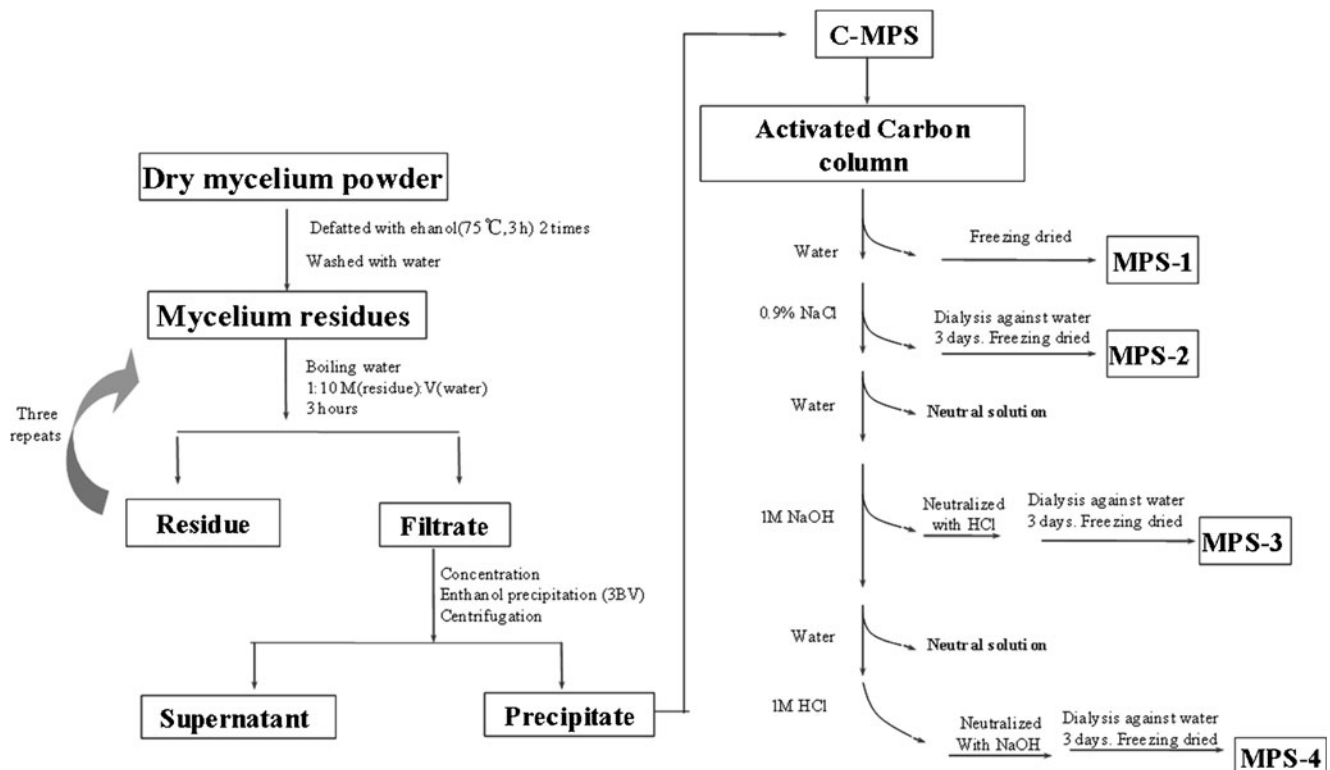
Fig. 1 Isolation process of polysaccharide fractions from *Tuber* fermentation mycelium by the activated carbon method. The combined aqueous extract of each residue was precipitated by adding 3BV ethanol filtered, and the precipitates dried to obtain crude mycelium polysaccharide (C-MPS). Mycelium polysaccharides-1 (MPS-1), mycelium polysaccharides-2 (MPS-2), mycelium polysaccharides-3 (MPS-3), and mycelium polysaccharides-4 (MPS-4) were obtained from crude mycelium polysaccharide (C-MPS) after gradient elution with water, 0.9 % NaCl, 1 M NaHO, and 1 M HCl in activated carbon column

ELISA Reader (Bio-TeK Instruments, Winoski, VT). The tested sample groups were compared with the negative control group. All in vitro results were expressed as the inhibition ratio of tumor cell proliferation, calculated using the following formula: Growth inhibition rate=(1−absorbance of experimental group/absorbance of negative control group)×100 %. All assays were carried out in triplicate.

Inhibition on HL-60 cell proliferation

HL-60 (1.0×10^4 cells/mL) were incubated in 96-well plates containing 90 μL of the culture medium at 37 °C in a humidified atmosphere with 5 % CO_2. A 10-μL aliquot of 52 polysaccharides at two final concentrations (20 and 200 μg/mL) in culture medium was added to each well. Samples were compared to a blank control group (10 μL of sample solution, no cells) and a negative control group (10 μL of 0.5 % DMSO). After 48 h of incubation at 37 °C, cells in each well were then incubated in culture medium with 20 μL of 5 mg/mL MTT solution for 3 h at 37 °C. Next, 120 μL of buffer solution (5 % SDS, 10 mM HCl, 5 % isopropyl alcohol) was added to each well. Absorbance at 595 nm was determined by a Power Wave X Micro-plate ELISA Reader (Bio-TeK Instruments, Winoski, VT). The tested sample groups were compared with the negative control group. All in vitro results were expressed as the inhibition ratio of tumor cell proliferation calculated using the formula: Growth inhibition rate= [1−(absorbance of experimental group−absorbance of blank control group)/(absorbance of negative control group−absorbance of blank control group)]×100 %. All assays were carried out in triplicate.

Chemical composition analysis

Five-milligram fractions of F-*T. m*-MPS-a and *T. m*-FPS-a were hydrolyzed with 2 M TFA at 100 °C for 6 h, dried using N_2, washed twice with methanol, and dried a second time by using N_2. The hydrolysis products were acetylized, as described by Englyst et al. (1994). Alditol acetate derivatives of each monosaccharide standard (D-glucose, D-mannose, D-galactose, D-fructose, and D-arabinose) and their mixtures were prepared. The fractions F-*T. m*-FPS-a, F-*T. m*-MPS-1, *T. m*-MPS-1, *T. m*-FPS-1, *T. i*-MPS-1, and *T. i*-FPS-1 were combined with 0.2 mL of 5 M HCl, vortexed, and then 50 mL of acidified ethanol was added. After mixing well, precipitates of each reaction system were cooled in ice water for 1 h, centrifuged at 11,000 rpm for 30 min (4 °C), and the residue was washed with 85 % and 100 % ethanol in turn. Each

enzymatic hydrolysate was further hydrolyzed and acetylized using the method used with the fractions F-*T. m*-MPS-a and *T. m*-FPS-a. All alditol acetate derivatives were analyzed by GC using a Varian 3400 instrument (Hewlett–Packard Component, USA) equipped with HP-5 column (50 mm× 0.20 mm×0.25 μm). The column temperature was kept at 120 °C for 2 min, and then increased to 250 °C for 3 min at a rate of 8 °C/min.

Molecular weight determination by GPC-ESLD

The molecular weights (M_W) of the fractions were determined by gel permeation chromatography (GPC). GPC was performed on a Waters HPLC instrument with an ultrahydrogel column (Sepax SRT SEC-500, 7.8 mm×300 mm), a Varian 380-LC Evaporative Light-scattering Detector, with water as the mobile phase at a flow rate of 0.1 mL/min. The samples were dissolved in water (0.5 mg/mL) and filtered through a 0.2-μm membrane prior to sample injection. The M_W of the samples were derived from the calibration curve measured with linear dextran M_W standards at 4.32×10^3, 1.26×10^4, 6.06×10^4, 11.0×10^4, and 28.9×10^4 g/mol (Chinese CRM/RM Information Center).

NMR analysis of the fractions F-T. m-MPS-a and F-T. m-MPS-1

^{1}H and ^{13}C NMR spectra of the fraction F-*T. m*-MPS-a were obtained in D_2O using a Mercury-300BB spectrometer (Varian, USA) at 30 °C. The fraction F-*T. m*-MPS-1, isolated from the same kind of mycelium by using a different method (activated carbon method), was partly hydrolyzed with 2 M TFA (80 °C, 3 h) and acetylized as described (Englyst et al. 1994) because of its poor solubility in water and DMSO. The ^{1}H and ^{13}C NMR spectra of the alditol acetate derivative from the fraction F-*T. m*-MPS-1 were obtained in dichloromethane (CH_2Cl_2).

FT-IR spectroscopy analysis

Infrared spectra (IR) of the samples were recorded with a Nicolet NEXUS 670 FT-IR (Spectrum One, PerkinElmer Co., USA) spectrometer in the range 4,000–500 cm^{-1} by using the KBr disk method.

Results

Polysaccharide fraction preparation

All of the crude polysaccharides were named as the form of "strain-synthetic route-extracted method". For example, "*T. i*" is the abbreviation of strain *T. indicum*. "BPS" represents the crude polysaccharide produced from the fruiting body (Table 1). "1" represents the crude polysaccharides "*T. i*-BPS-1" was purified and separated with H_2O by activated carbon column method (Table 1). So, the crude polysaccharide was named "*T. i*-BPS-1".

Crude polysaccharides of the *Tuber* fermentation mycelia and the natural fruiting bodies were extracted by the activated carbon column procedure to compare their nutritional qualities. F-*T. m*-FPS-a, F-*T. m*-FPS-b, F-*T. m*-FPS-c, and *T. m*-FPS-a were initially obtained before the activated carbon purification methods were adopted. An in vitro antitumor assay indicated that the fraction F-*T. m*-FPS-a extracted by purified water, exhibited a relatively higher cell growth inhibition rate. According to the results of the in vitro antitumor assay, water extracts of *Tuber* fruiting bodies and fermentation mycelium were chosen as the unique source of crude polysaccharides during sample preparation. Crude polysaccharides *T. indicum* fruiting body polysaccharides (*T. i*-BPS), *T. himalayense* fruiting body polysaccharides (*T. h*-BPS), *T. sinense* fruiting body polysaccharides (*T. s*-BPS) from *T. indicum*, *T. himalayense*, and *T. sinense* fruiting bodies were obtained by hot water extraction and ethanol precipitation (Sevag 1934). The results showed that the commonly used Sevag's procedure was capable of purifying crude polysaccharides from the fermentation systems of *T. melanosporum*, which contain little pigment, but did not work for those from the *Tuber* fruiting bodies, which contain massive amounts of pigment. To solve this problem, activated carbon was successfully used to discolor the crude polysaccharides. Subsequently, the polysaccharide and protein assays indicated that all fractions washed from the activated carbon column were polysaccharide solutions free from protein. A total of 52 polysaccharide fractions were isolated by this activated carbon method (Table 2).

In vitro antitumor activity

The HepG2, A549, HCT-116, SK-BR-3, and HL-60 cells were treated with the 52 polysaccharide fractions at the concentrations of 20 and 200 μg/mL, respectively. Among the 260 (52×5) total experimental groups, 226 groups exhibited obvious dose-dependent behavior, that is, the majority of polysaccharide fractions exhibited higher inhibition at 200 μg/mL than at 20 μg/mL (Table 2); therefore, the data obtained at 200 μg/mL were analyzed first.

Among the 52 fractions, the inhibition rates of 12 fractions (at least on one kind of cell) were greater than 50 % (Table 2). Among these 12 fractions, only two were isolated from *Tuber* fruiting bodies, *T. i*-BPS-3 and *T. i*-BPS-4, while the others were all isolated from fermentation systems. This indicated that the polysaccharides from the *Tuber* fermentation systems exhibited higher in vitro antitumor activity than fruiting bodies. In addition, 10 of the 12 fractions inhibited the growth of

Table 2 Inhibition rate of 52 truffle polysaccharide fractions on five kinds of tumor cells in 72 h

Sample	Inhibition rate (%)									
	HCT-116		Sk-BR-3		A549		HL-60		HepG2	
	20 μg/mL	200 μg/mL	20 μg/mL	200 μg/mL	20 μg/mL	200 μg/mL	20 μg/mL	200 μg/mL	20 μg/mL	200 μg/mL
T. i-BPS-1	5.05±0.85	5.76±1.12	1.52±0.42	19.94±2.32	−13.11±3.14	1.14±0.07	3.87±3.23	4.15±6.74	18.12±3.31	26.58±8.54
T. i-BPS-2	−2.45±0.77	27.93±3.65	1.13±0.31	2.55±1.55	−16.74±2.14	9.03±3.11	4.12±1.41	16.73±5.55	6.25±2.43	49.21±2.24
T. i-BPS-3	3.53±1.02	38.13±4.22	0.96±0.15	32.64±6.01	3.15±2.44	19.70±5.21	0.09±0.02	24.06±3.57	25.36±2.87	58.97±5.34
T. i-BPS-4	−0.25±0.07	5.02±1.05	1.29±0.51	22.99±5.13	7.47±3.30	20.45±3.33	−1.96±0.41	22.77±4.18	41.07±3.33	50.70±4.34
T. s-BPS-1	3.59±0.13	5.21±1.85	6.33±1.35	32.00±4.62	−14.10±2.32	2.75±1.10	−13.47±2.23	−1.50±0.04	−16.38±1.87	16.20±1.11
T. s-BPS-2	1.26±0.87	4.02±2.32	0.91±0.32	1.83±0.32	−13.08±4.32	−8.11±2.21	−6.35±1.82	−0.94±0.11	−11.68±2.22	−2.90±0.03
T. s-BPS-3	−6.44±1.89	−1.32±0.45	−4.28±1.25	15.22±2.99	−12.48±0.91	−16.97±4.98	−10.91±2.90	−8.40±2.14	−16.40±6.12	−8.45±2.43
T. s-BPS-4	4.10±0.44	0.56±0.05	3.87±1.98	2.36±1.02	−11.95±4.21	−6.98±0.88	−10.03±2.33	−2.38±1.21	−8.52±3.14	−0.33±0.07
T. h-BPS-1	12.78±2.85	16.32±1.93	11.39±3.95	41.93±5.92	5.05±1.13	8.17±3.23	0.03±0.01	25.62±4.57	11.02±5.87	22.80±3.76
T. h-BPS-2	7.41±1.65	9.08±2.99	−7.65±1.43	−4.00±1.00	−1.57±0.31	1.39±0.41	0.23±0.08	2.44±1.22	−5.90±2.91	6.42±2.33
T. h-BPS-3	4.89±2.14	4.19±1.97	−3.93±1.44	0.36±0.02	2.39±0.43	−1.88±0.02	0.33±0.03	1.09±0.22	−7.23±1.24	4.17±0.32
T. h-BPS-4	−1.18±0.33	−3.54±0.02	7.09±3.98	0.30±0.12	−10.26±0.21	−3.20±1.24	−9.80±3.58	−13.63±1.93	−7.17±2.23	−1.56±0.69
T. m-FPS-1	16.43±3.25	47.34±2.56	0.90±0.15	26.27±6.55	29.80±4.23	34.28±8.77	−0.76±0.01	15.17±4.66	22.64±3.23	65.85±5.88
T. m-FPS-2	−3.51±0.49	15.17±3.33	1.09±0.62	27.90±1.54	−3.28±1.91	28.89±9.53	0.62±0.91	13.20±3.12	19.29±6.12	51.53±4.40
T. m-FPS-3	16.18±1.66	37.66±1.67	42.18±6.32	62.12±0.12	14.74±5.08	21.07±2.95	−0.73±0.27	17.02±0.67	38.18±4.31	64.79±6.43
T. m-FPS-4	−1.10±0.88	38.20±4.32	1.11±0.81	30.98±9.24	−5.33±0.43	17.72±4.76	3.03±0.88	20.92±1.12	7.29±2.31	40.84±3.26
F-T. m-MPS-a	−9.60±2.21	23.30±3.24	1.22±0.36	−2.74±0.54	−21.99±5.43	17.19±3.53	0.84±0.91	13.04±2.36	3.62±1.91	44.43±2.46
F-T. m-MPS-b	−6.67±1.46	5.17±2.54	1.26±0.23	21.09±8.33	−20.41±0.22	−4.44±2.21	2.92±0.77	−0.04±0.01	7.75±3.21	20.08±3.24
F-T. m-MPS-c	−9.07±2.26	−2.88±0.15	1.07±0.52	15.36±2.12	−6.80±2.92	−2.71±0.79	2.49±0.87	3.19±1.24	−2.80±0.32	12.29±2.37
F-T. m-MPS-1	6.65±3.01	52.02±5.81	1.11±0.21	43.36±6.32	−15.31±5.32	58.60±5.36	0.70±0.02	31.25±3.65	12.80±2.71	69.18±5.32
F-T. m-MPS-2	−3.05±1.33	12.46±2.61	1.06±0.51	31.04±5.36	−8.92±3.35	21.46±4.32	0.47±0.31	0.69±0.12	−18.24±3.33	32.87±3.63
F-T. m-MPS-3	−7.06±2.22	62.82±4.33	1.20±0.17	43.39±7.15	−14.78±2.36	50.72±3.22	4.55±1.91	31.64±0.65	−10.90±2.14	69.95±6.43
F-T. m-MPS-4	−7.95±1.43	−5.00±1.15	−4.48±0.13	−4.35±1.66	−4.96±1.35	−4.77±1.21	−0.10±0.01	−2.23±0.11	−6.43±3.67	−5.57±1.24
T. m-FPS-a	−5.14±2.37	−5.49±0.81	0.96±0.05	13.24±2.67	12.31±2.56	7.75±2.44	18.26±4.87	4.46±0.79	−7.07±5.98	12.68±0.91
T. m-MPS-1	15.48±3.35	18.14±4.92	9.32±0.16	21.72±2.41	2.10±0.96	6.78±2.26	−9.50±3.22	6.31±1.57	12.17±2.94	9.10±1.24
T. m-MPS-2	5.70±0.91	9.01±2.67	−3.46±0.21	1.35±0.65	−3.70±0.91	−1.71±0.62	−15.14±0.45	−9.10±1.24	−2.07±0.46	0.64±0.08
T. m-MPS-3	3.37±1.45	10.73±3.66	13.00±0.80	30.75±5.17	−7.61±2.34	8.69±3.32	−17.97±2.34	−14.72±2.23	−3.10±1.23	10.50±2.34
T. m-MPS-4	−5.05±0.38	0.53±0.13	1.14±0.66	0.80±0.09	−6.36±1.25	−7.50±2.21	−8.03±3.23	−7.18±3.12	0.19±0.03	3.16±1.13
T. i-MPS-1	12.04±2.88	19.09±4.24	7.97±2.45	31.51±6.17	−7.36±0.54	4.42±0.57	−1.41±0.93	−6.39±0.43	−8.14±1.92	1.84±0.54
T. i-MPS-2	−4.97±1.15	−9.45±1.25	3.07±0.85	8.04±3.16	−28.31±2.35	−28.43±1.21	−13.08±3.21	−9.91±1.76	−11.75±2.38	−1.66±0.33
T. i-MPS-3	2.79±0.44	0.81±0.09	4.96±1.11	39.77±8.21	−18.16±6.32	−13.99±0.91	−11.52±0.31	−9.34±1.67	−10.78±2.17	9.66±3.23
T. i-MPS-4	1.30±0.72	4.19±0.32	1.09±0.34	5.99±1.43	−21.09±7.33	−18.62±6.09	−13.36±3.92	−8.10±2.13	−9.49±2.44	−4.10±1.58

Table 2 (continued)

Sample	Inhibition rate (%)									
	HCT-116		Sk-BR-3		A549		HL-60		HepG2	
	20 μg/mL	200 μg/mL	20 μg/mL	200 μg/mL	20 μg/mL	200 μg/mL	20 μg/mL	200 μg/mL	20 μg/mL	200 μg/mL
T. i-FPS-1	16.56±3.25	0.97±0.21	25.88±0.65	68.11±5.65	−4.78±1.45	−0.39±0.11	−6.57±3.11	2.36±0.21	26.16±5.38	24.80±5.32
T. i-FPS-2	−4.82±0.85	−5.13±1.25	4.14±0.09	11.76±3.13	−24.61±5.24	−19.02±6.16	2.78±0.94	−1.88±0.35	−5.20±1.83	7.20±2.36
T. i-FPS-3	6.97±2.43	9.26±2.24	10.97±0.14	36.40±1.37	−14.88±3.44	−6.54±2.36	−0.86±0.22	−1.91±0.64	−1.51±0.74	2.77±0.53
T. i-FPS-4	5.67±1.65	0.01±0.00	−2.54±1.85	23.58±5.17	−6.24±2.35	−7.65±2.21	−4.71±2.42	−3.21±1.98	6.74±2.87	9.54±2.12
T. s-MPS-1	−7.59±2.16	2.55±0.05	1.07±0.34	17.45±8.33	−10.55±4.35	4.20±0.87	5.09±5.32	2.47±0.43	22.64±4.90	22.86±5.37
T. s-MPS-2	2.30±0.35	2.25±0.27	6.97±1.22	5.75±2.32	−21.72±3.80	−15.35±4.21	−1.54±0.77	−14.00±8.11	1.15±0.61	7.36±2.94
T. s-MPS-3	14.25±2.55	36.63±5.42	47.31±5.17	62.53±4.76	6.95±2.34	21.49±6.43	−13.46±4.21	25.69±6.31	35.84±6.32	66.16±6.35
T. s-MPS-4	13.55±0.85	10.47±1.36	5.79±1.85	17.48±2.43	−4.82±0.91	−6.39±3.00	7.97±2.02	0.27±0.10	1.19±0.63	12.70±2.14
T. s-FPS-1	10.86±3.62	13.27±2.24	15.77±0.87	26.77±5.37	−15.06±0.55	5.47±0.37	−9.76±0.12	7.29±1.24	4.50±0.91	10.01±3.89
T. s-FPS-2	−1.63±0.44	−1.37±0.36	4.28±1.98	13.03±5.21	−21.28±3.79	−16.87±6.21	−5.28±0.91	−0.95±0.61	−0.15±0.09	9.90±2.12
T. s-FPS-3	3.82±0.15	1.91±0.21	5.42±1.45	13.62±7.18	−16.44±5.32	−10.99±2.32	−4.20±0.14	−7.81±3.11	2.77±1.01	−2.02±0.21
T. s-FPS-4	4.02±1.27	1.00±2.26	33.00±7.76	52.62±6.77	23.88±3.76	17.60±3.21	−5.01±1.81	−1.50±0.98	38.05±0.91	45.76±3.76
T. a-MPS-1	2.67±0.11	10.63±2.74	19.51±2.92	35.60±2.22	−4.94±0.31	11.01±3.22	11.69±3.91	−0.94±0.37	3.85±1.13	24.26±4.23
T. a-MPS-2	−4.56±1.85	−47.16±5.31	36.83±3.32	69.24±3.46	24.13±5.87	−13.34±4.32	−21.65±0.25	−13.86±3.91	\	\
T. a-MPS-3	−7.12±2.45	−7.93±1.98	−9.85±3.21	14.28±2.55	−6.32±0.21	−8.20±1.24	−1.34±1.76	−6.15±2.25	12.03±2.42	−10.27±3.23
T. a-MPS-4	−2.61±0.13	−3.68±1.65	−0.26±0.04	−2.62±0.32	−4.52±1.12	−0.62±0.08	−22.76±3.13	−12.98±1.65	−10.26±4.32	2.03±0.18
T. a-FPS-1	0.27±0.05	−15.14±4.98	2.70±0.32	−0.34±0.02	2.55±1.08	23.06±2.34	−10.84±4.12	−21.29±6.01	−15.58±2.34	17.69±0.52
T. a-FPS-2	6.02±0.44	−35.52±2.33	−8.79±1.13	3.31±1.11	11.75±0.32	28.83±5.32	−6.65±1.09	−6.42±2.71	2.18±0.84	33.52±2.63
T. a-FPS-3	5.70±2.21	38.27±6.01	36.32±7.33	63.90±6.12	24.37±3.92	30.77±1.35	15.91±6.21	10.33±1.39	22.99±3.15	54.27±5.34
T. a-FPS-4	−0.47±0.11	8.90±1.33	33.74±8.01	50.51±4.77	21.56±5.08	18.55±3.20	0.01±0.00	12.88±2.22	37.44±4.87	28.47±2.12

HepG2 cells; 7 fractions inhibited the growth of Sk-Br-3 cells; 2 fractions inhibited the growth of HTC-116 cells; 2 fractions inhibited the growth of A549 cells; and no fraction inhibited the growth of HL-60 cells (Table 2). This indicated that *Tuber* polysaccharides exhibited stronger effects on the growth of HepG2 cells compared to the other four types of cell lines.

A total of 10 polysaccharide fractions obtained from fermentation systems were found to exhibit higher inhibition rates (>50 %) on HepG2 cells of these, 5 fractions were isolated from *T. melanosporum*; 2, from *T. indicum*; 2, from *T. sinense*; and 2, from *T. aestivum* (Table 2). This indicated that *T. melanosporum* fermentation system polysaccharides exhibited stronger effects on the growth of HepG2 cells compared to the polysaccharides from the other three *Tuber* species.

Among the polysaccharide fractions isolated from *T. melanosporum* fermentation systems, the HepG2 cell growth inhibition rates of the fractions F-*T. m*-MPS-1, F-*T. m*-MPS-3, and *T. m*-FPS-1, which were isolated by the activated carbon method, were 69.18, 69.95, and 65.85 %, respectively. Conversely, the fractions F-*T. m*-MPS-a, F-*T. m*-MPS-b, F-*T. m*-MPS-c, and *T. m*-FPS-a, which were isolated by traditional methods, exhibited low inhibition rates of 44.43, 20.08, 12.29, and 12.68 %, respectively.

Structural analysis

Four fractions from the *T. melanosporum* fermentation systems were selected for further analysis based on our observations of cell growth inhibition and efficiency of the isolation process. The infrared (IR) spectra of all 4 fractions (Fig. 2) displayed broad intense peaks at 3,396 cm^{-1}, 3,408 cm^{-1}, 3,406 cm^{-1}, and 3,419 cm^{-1} for the hydroxyl and amine groups, and weak C–H stretching bands at 2,927 cm^{-1}, 2,927 cm^{-1}, 2,926 cm^{-1}, and 2,925 cm^{-1}, respectively. Further, an asymmetrical stretching peak was observed between 1,622 and 1,654 cm^{-1} and a weak symmetrical stretching peak near 1,409–1,426 cm^{-1}, suggesting the presence of carboxyl groups. In addition, the peaks in the 1,108–1,142 cm^{-1} range of the four fractions indicated the presence of glucuronic acid (Gonzaga et al. 2005). F-*T. m*-MPS-a and F-*T. m*-MPS-1 exhibited absorption peaks at 876, 812, 874, and 810 cm^{-1} for mannan, consistent with results of a previous study (Mathlouthi and Koenig, 1986). As shown in Fig. 2, the IR absorption of F-*T. m*-MPS-1 at 818 cm^{-1} is the characteristic absorption of galactose. The presence of proteins was confirmed in F-*T. m*-MPS-1 by IR absorptions at 3546, 1684, and 669 cm^{-1} corresponding to the protein CONH– groups. The coexistence of α- and β-D-glucans was confirmed in *T. m*-FPS-a and *T. m*-FPS-1 by the appearance of characteristic peaks at 859, 915, 864, and 916 cm^{-1} for α-D-glucan, and at 883 and 895 cm^{-1} for β-D-glucan, respectively. In *T. m*-FPS-a, the absorption peaks at 829 and 803 cm^{-1} were observed for
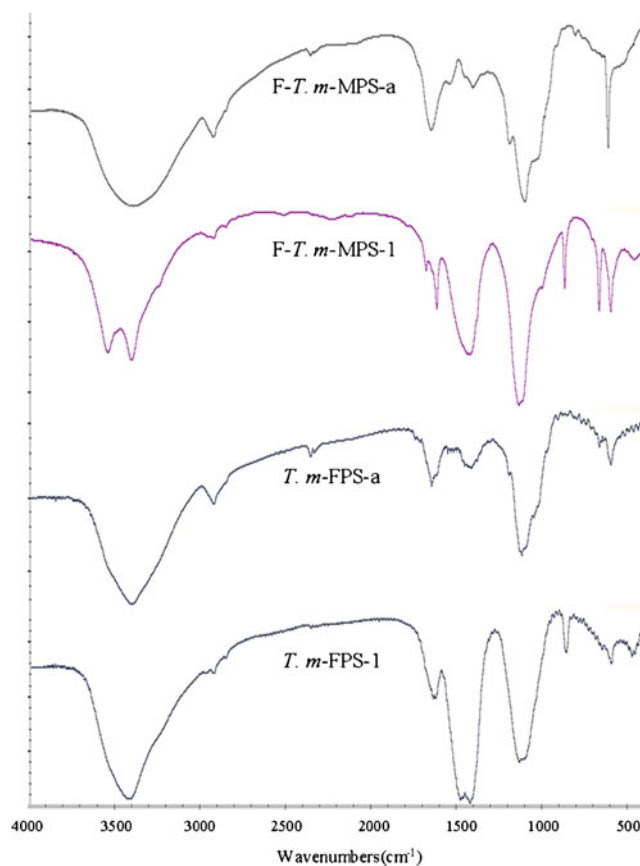

Fig. 2 FT-IR spectra of *T. melanosporum* fermentation mycelium polysaccharides-a (*F-T. m-MPS-a*), *T. melanosporum* fermentation mycelium polysaccharides-a (*F-T. m-MPS-1*), *T. melanosporum* batch fermentation broth polysaccharides-a (*T. m-FPS-a*), and *T. melanosporum* batch fermentation broth polysaccharides-a (*T. m-FPS-1*) polysaccharide fractions from *T. melanosporum* fermentation system. All four polysaccharide contain the hydroxyl, the amine groups, glucuronic acid, carboxyl groups and weak C–H stretching bands

α-galactose and mannose, respectively. The IR spectra (Fig. 2) further revealed the existence of proteins in *T. m*-FPS-1 based on the absorption peaks at 1880 and 669 cm^{-1}, and mannan as evidenced by the absorptions at 865 and 810 cm^{-1}.

As shown in Fig. 3, the F-*T. m*-MPS-a, *T. m*-FPS-a, and monosaccharide standards were first hydrolyzed by TFA, and their acetate derivatives were detected. Compared with the monosaccharide standards (D-glucose, D-mannose, D-galactose, D-xylose, D-arabinose, and L-rhamnose), the peaks of F-*T. m*-MPS-a and *T. m*-FPS-a acetate derivatives were identified as D-mannose, D-glucose, and D-galactose. The other five fractions were also hydrolyzed by TFA, but no characteristic peaks were observed (data not shown). Therefore, those fractions and the monosaccharide standards were simultaneously hydrolyzed by TFA and cellulose. To confirm the feasibility of the latter method, *T. m*-FPS-a was treated using the same method. The results of this experiment indicated that the new hydrolysis method was viable for all samples, except

Fig. 3 GC analysis of *Tuber* polysaccharide acetate derivatives hydrolyzed by TFA. (A) The chromatogram of monosaccharide standards (D-glucose, D-mannose, D-galactose, D-xylose, D-arabinose, and L-rhamnose), (B) the chromatogram of *T. melanosporum* batch fermentation broth polysaccharides-a (*T. m*-FPS-a) sample. (C) the chromatogram of *T. melanosporum* fermentation mycelium polysaccharides-a (F-*T. m*-MPS-a)
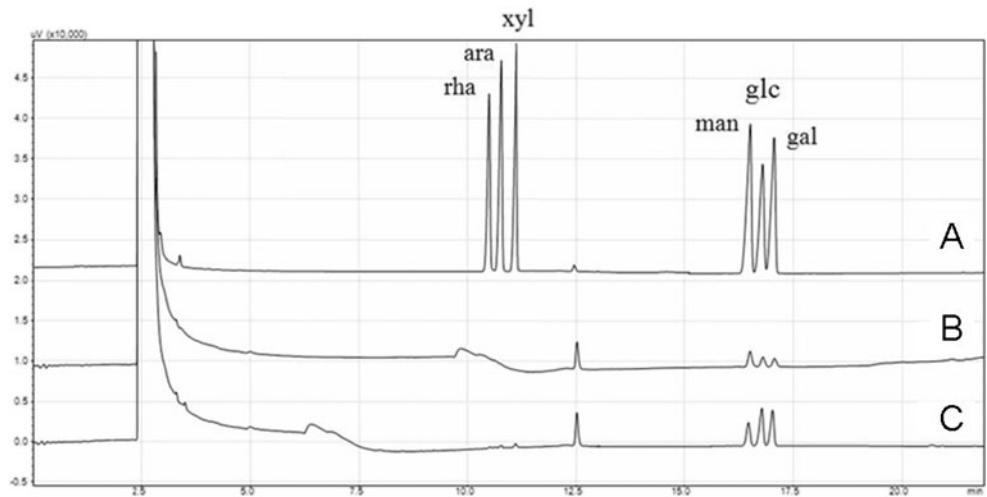
for the monosaccharide standards (Fig. 4). Compared with *T. m*-FPS-a, the peaks observed for acetate derivatives of F-*T. m*-MPS-1, *T. m*-FPS-1, *T. i*-BPS-1, *T. i*-MPS-1, and *T. i*-FPS-1 were identified as D-mannose, D-glucose, and D-galactose. The relative contents of D-glucose in the F-*T. m*-MPS-a, F-*T. m*-MPS-1, *T. m*-FPS-a, and *T. m*-FPS-1 were 43.29, 39.08,

Fig. 4 GC analysis of *Tuber* polysaccharide acetate derivatives hydrolyzed by TFA and cellulase. The chromatogram *a*, *b*, *c*, *d*, *e*, *f*, *g*, and *h* represented the chromatogram of *T. indicum* fermentation mycelium polysaccharides-1 (*T. i*-MPS-1), *T. indicum* fermentation broth polysaccharides-1 (*T. i*-FPS-1), *T. melanosporum* batch fermentation broth polysaccharides-1 (*T. m*-FPS-1), *T. melanosporum* batch fermentation mycelium polysaccharides-1 (*T. m*-MPS-1), *T. melanosporum* fermentation mycelium polysaccharides-1 (F-*T. m*-MPS-1), *T. indicum* fruiting body polysaccharides-1 (*T. i*-BPS-1), *T. melanosporum* batch fermentation broth polysaccharides-a (*T. m*-FPS-a), *T. indicum* fermentation broth polysaccharides-1 (*T. i*-FPS-1)
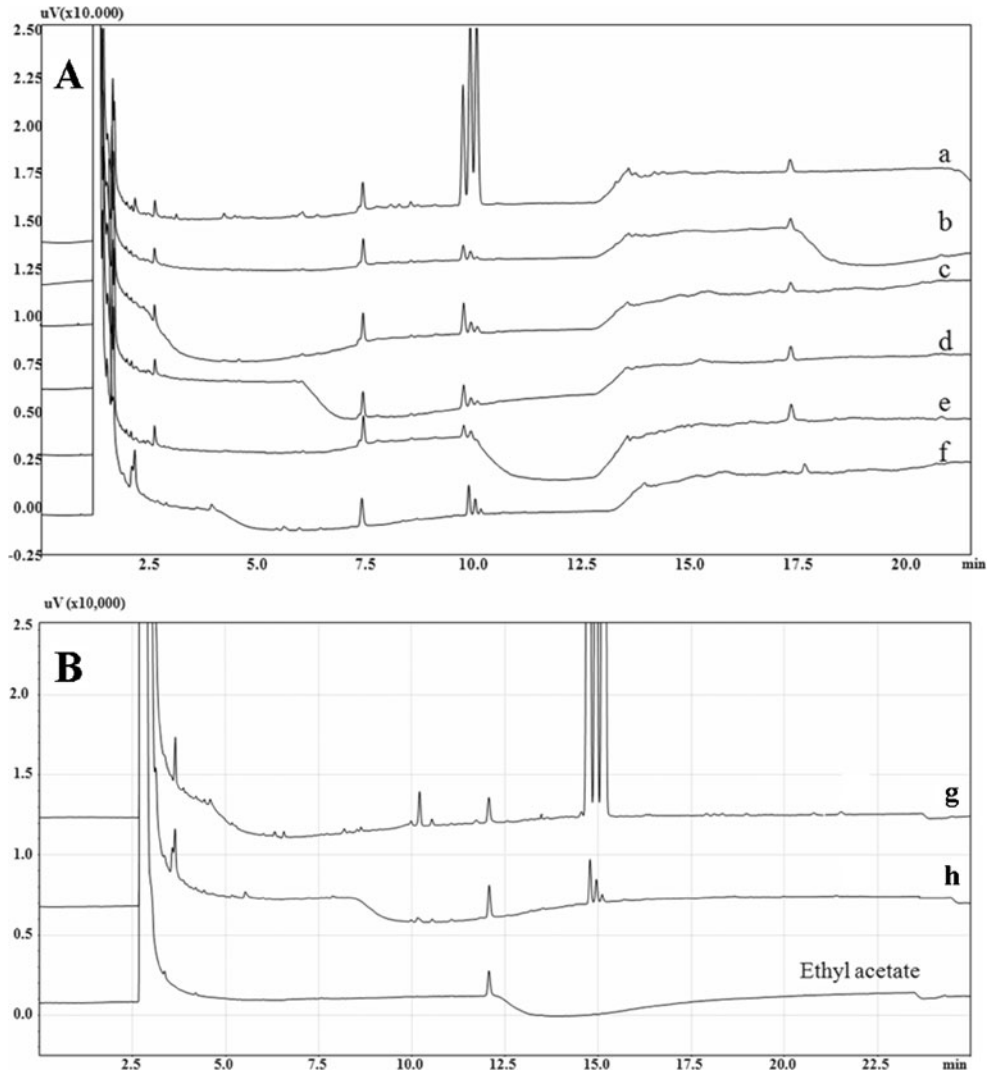
Table 3 M_W value of polysaccharide fractions

Polysaccharides	M_W value of each fraction				
T. i-BPS-1	1.36×10^5	6.18×10^4	1.68×10^4	8.2×10^3	3.63×10^3
	(32.9)	(35.2)	(40.5)	(43.4)	(46.7)
T. i-MPS-1	4.5×10^6	3.92×10^5	1.26×10^5	–	–
	(17.8)	(27.7)	(32.3)		
T. i-FPS-1	8.33×10^6	6.00×10^4	2.37×10^4	1.31×10^4	4.9×10^3
	(15.3)	(35.3)	(39.1)	(41.5)	(45.5)
F-T. m-MPS-1	2.74×10^4	1.22×10^4	3.34×10^3	–	–
	(38.5)	(41.8)	(47)		
F-T. m-MPS-a	8.97×10^6	1.05×10^5	–	–	–
	(15.0)	(33.0)			
T. m-FPS-1	1.68×10^6	1.66×10^5	8.95×10^4	6.5×10^4	1.16×10^4
	(21.8)	(31.2)	(33.7)	(35.0)	(42.0)
T. m-FPS-a	4.08×10^6	1.16×10^6	5.7×10^3	5.0×10^3	3.2×10^3
	(18.2)	(23.3)	(44.9)	(45.4)	(47.2)

23.75, and 0.25 %, respectively. The relative contents of D-mannose in F-T. m-MPS-a, F-T. m-MPS-1, T. m-FPS-a, and T. m-FPS-1 were 39.24, 59.19, 60.08, and 0.63 %, respectively. The relative contents of D-galactose in the F-T. m-MPS-a, F-T. m-MPS-1, T. m-FPS-a, and T. m-FPS-1 were 17.74, 1.72, 15.45, and 0.12 %, respectively. Furthermore, the results of the M_W value of polysaccharide fractions were summarized in

Table 3. It was noted that a too-high M_W value may weaken the antitumor activity.

The ^{1}H NMR spectrum of the F-T. m-MPS-1 (Fig. 5A) revealed 4 signals (i.e., δ 5.111 and 5.184) in the region for anomeric protons, indicating pentasaccharide repeating units. In addition, in the ^{13}C NMR spectrum of F-T. m-MPS-1 (Fig. 5B), the anomeric region (109–81 ppm) showed four

Fig. 5 NMR spectrum of T. melanosporum fermentation mycelium polysaccharides-a (F-T. m-MPS-1). (A) The ^{1}H NMR spectrum of the F-T. m-MPS-1. (B) The ^{13}C NMR spectrum of F-T. m-MPS-1
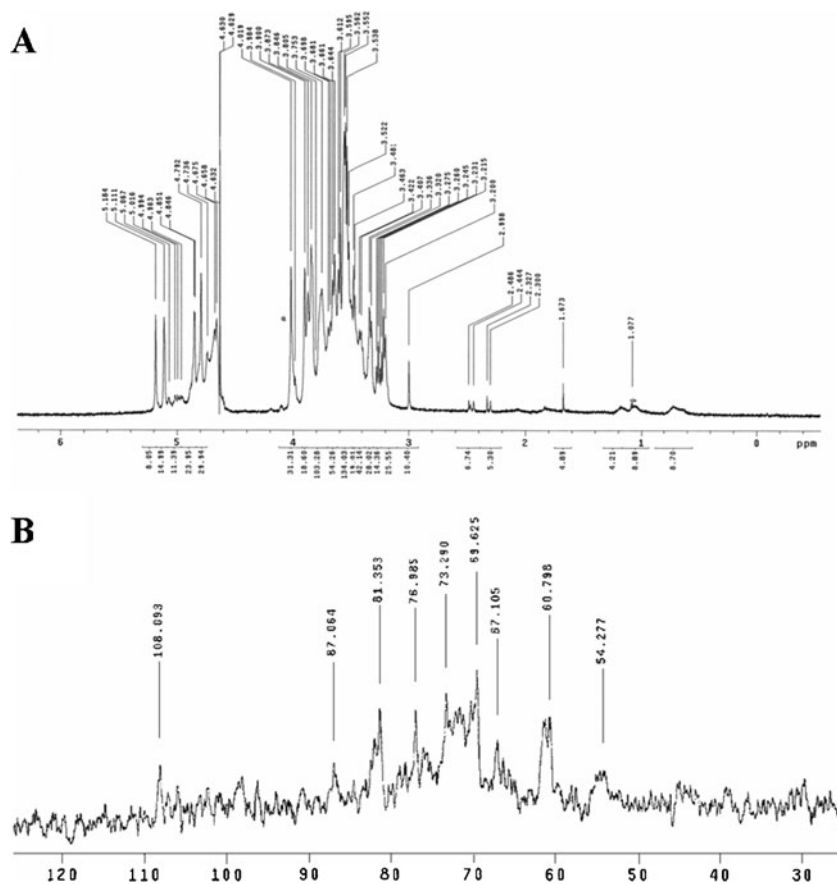
main signals at 108.1 and 87.1 ppm in F-*T. m*-MPS-1, which were assigned as the C-1 of glucose, galactose, *O*-methyl-galactose, and mannose, respectively. We did not obtain signals between 108 and 88 ppm; therefore, we ruled out the presence of 2-*O*-, 6-*O*-, and 4-*O*-methyl-galactose; however, the high signal near 54 ppm indicated the presence of 3-*O*-methyl-galactose. After sulfation, peaks in the ^{13}C NMR spectra became more difficult to assign, because the carbon directly attached to the electron-withdrawing sulfate group shifted to a lower field position, while the carbon indirectly attached to the sulfate group shifted to a higher field position. The spectrum for F-*T. m*-MPS-1 showed a split in the signals at 81–87 ppm for C-1. It is known that the signal of C-1 splits when an OH group on C-2 is functionalized, and this splitting of the C-1 signal correlated well with the extent of substitution of C-2. In addition, the disappearance of the signal near 69.6 ppm indicated that C-2 of one of the monosaccharides was completely sulfated. The new peaks in the 76.9-ppm region of F-*T. m*-MPS-1 were assigned to the O-4 substituted carbons, suggesting that O-4 was sulfated. In view of the intensities of the signals of the O-substituted carbons, we conclude that nonselective sulfation of the extracellular polysaccharide occurred; in particular, C-2 and C-4 were partially substituted, whereas C-6 was not affected.

Discussion

Edible mushroom consumption is associated with the improvement of human health, especially for cancer prevention. Interestingly, the active constituents that are involved in these effects of mushrooms are considered to be polysaccharides, which can induce tumor cell death in several cancer types. In the present study, our development of the activated carbon method to resolve the problem of hardly pigment resulted in the effective isolation of 52 polysaccharides from the *T. melanosporum*, *T. indicum*, *T. sinense*, and *T. aestivum* fermentation systems for the first time. Based on a comparison of the chemical compositions, molecular weight, and ^{13}C NMR results, we identified 4 *Tuber* polysaccharides (i.e., F-*T. m*-MPS-a, F-*T. m*-MPS-1, *T. m*-FPS-a, and *T. m*-FPS-1) in *Tuber* fruiting bodies and fermentation systems. Both F-*T. m*-MPS-a and F-*T. m*-MPS-1 were found to be mannan. Polysaccharides from *T. m*-FPS-a and *T. m*-FPS-1 were found to contain both α- and β-D-glucans, while those from F-*T. m*-MPS-1 and *T. m*-FPS-1 were found to contain protein groups. All polysaccharides were mainly composed of D-mannose, D-glucose, and D-galactose, which suggests that all polysaccharides from *Tuber* fruiting bodies and fermentation systems have identical chemical composition. To compare the biological activities of the fractions, we evaluated antitumor effects. The in vitro antitumor assay indicated that the polysaccharides from *Tuber* fermentation exhibited a relatively higher cell growth inhibition rate than those from fruiting bodies. The maximal inhibition rate of polysaccharides from *Tuber* fermentation was 69.95 % (i.e., F-*T. m*-MPS-3), which was approximately 16 % greater than that (i.e., *T. i*-BPS-3, 58.97 %) from the fruiting bodies.

It has also been suggested that mushroom polysaccharides with high antitumor activities are mostly heteropolysaccharides (Cooi and Liu. 2000). Our results indicated the lower molecular weight of F-*T. m*-MPS-1 (Table 3), as acidic heteropolysaccharide, was shown to have the highest antitumor activity in polysaccharide fractions. But the higher molecular weight of F-*T. m*-MPS-a (Table 3) was shown to have the low antitumor activity (Table 2). The results of this study revealed that the antitumor activities of the polysaccharides strongly related to the molecular weight of the bound protein and chain conformation. It is widely recognized that the monosaccharide composition is considered as another important factor related to the anti-cancer activities of natural polysaccharides. The results of an in vitro assay (Table 2) indicated that the antitumor activities against HepG2 tumor cells of the polysaccharides from *Tuber* fruiting bodies were significantly lower than that of the polysaccharides from *Tuber* fermentation systems, which contain the triple helix conformation of β-D-glucan. The antitumor activities for the polysaccharides were found to be similar to those of native β-glucan, while the single-chain β-glucans exhibited no significant antitumor activity. Therefore, the triple helix conformation of β-D-glucan appears to play an important role in the enhancement of antitumor activities, and this may be further enhanced as a result of the protein bound to the polysaccharides.

It is notable that introduction of sulfate and carboxymethyl groups significantly increased the antitumor activity of β-glucan. Those polysaccharides (i.e., *T. m*-FPS-1) exhibited increased antitumor activities (Table 2). It has been reported that periodate modification and mild hydrolysis of the β-D-glucan of *P. cocos* sclerotium decreases the M_W and increases the water solubility of the glucan, resulting in enhancement of antitumor activity (Wang et al. 2004). Furthermore, some β-glucans have triple helix structures that exhibit distinct antitumor activities because of their stiff chain conformation (Zhang et al. 2001).

In conclusion, this is the first study to show that *Tuber* polysaccharides from a *Tuber* fermentation system exhibited relatively higher in vitro antitumor activity against HepG2, A549, HCT-116, SK-BR-3, and HL-60 cells than those from *Tuber* fruiting body. In total, 52 polysaccharides were isolated from *T. melanosporum*, *T. indicum*, *T. sinense*, *T. aestivum* fermentation systems and *T. indicum*, *T. himalayense T. sinense* fruiting body by elution with an activated carbon column. The observed antitumor effect indicates high-chain stiffness enhances antitumor activity and the *Tuber* polysaccharides with lower M_W values exhibited higher antitumor

activities. Among 12 polysaccharide fractions with inhibition rates greater than 50 %, ten polysaccharide fractions derived from the *Tuber* fermentation system. This work partly confirms the rationality of *Tuber* fermentation as an alternative resource for truffle fruiting bodies. The study indicates the possibility that *Tuber* fermentation may provide an effective and alternative strategy for an alternative resource for truffle fruiting bodies.

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