Identification and Antiproliferative Activity of Polysaccharides from *Tricholoma matsutake* (mushroom)

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Abstract: *Tricholoma matsutake*,also known as "the king of mushrooms," contains polysaccharides that had been reported to have anti-cancer, anti-radiation, and immune-enhancing effects. In this study, polysaccharides from *Tricholoma matsutake* (TM-P) were isolated and purified using a diethylaminoethyl (DEAE) Sepharose fast-flow column and a Sephadex G-75 column, which resulted in four purified fractions: TM-P1A, TM-P1B, TM-P2A, and TM-P2B. Structural characterization of these polysaccharide fractions was performed using gas chromatography-mass spectrometry (GC-MS),high-performance gel permeation chromatography (HP-GPC), and nuclear magnetic resonance (NMR) spectra. The results showed that the main components of TM-P1A and TM-P2B were glucose and galactose with a small amount of mannose in molar ratios of 8.7:1.8:1.0 and 8.9:1.3:1.0, respectively. TM-P2A was composed of glucose, galactose, mannose, and fucose, with a molar ratio of 17.7:7.9:3.9:1.0. The main component of TM-P2B was β-1,6-glucopyranose (unbranched) with a small amount of β-1,6-galactopyranose and β-1,6-mannopyranose. Among all fractions, TM-P2B showed the strongest antioxidant and antiproliferative activities. The oxygen radical absorbance capacity of TM-P2B was 2475.33±15.32μmolTrolox/g. At a concentration of 1.0mg/mL, TM-P2B showed 90.23% ± 2.17% inhibition of HepG2 human hepatoma cells, which was 1.2 and 3.1 times that observed with TM-P2A and TM-P1A, respectively. This study provides important information for further application and development of *Tricholoma matsutake*and related products.

Key words: *Tricholoma matsutake*; polysaccharides; purification; antioxidant activity; antiproliferative activity

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松茸多糖的结构分析及抗增殖活性研究

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摘要：松茸，被誉为"菌中之王"。多糖作为其重要的活性成分之一，被报道具有抗癌、抗辐射、提高免疫等功效。本研究采用琼脂糖凝胶柱 DEAE Sepharosse fast flow、葡聚糖凝胶柱 Sephadex G-75 对松茸多糖进行分离纯化，得到四个组分，分别为 TM-P1A、TM-P1B、TM-P2A 和 TM-P2B。再通过 GC-MS、HP-GPC 以及核磁共振波谱对其结构进行分析。结果表明 TM-P1A 和 TM-P2B 主要由葡萄糖和半乳糖组成，并含有少量甘露糖，其摩尔比分别为 8.7:1.8:1.0 和 8.9:1.3:1.0。TM-P2A 由葡萄糖、半乳糖、甘露糖和岩藻糖组成，摩尔比为 17.7:7.9:3.9:1.0。TM-P2B 主要由 β-1,6-吡喃型葡萄糖构成，无支链，有少量 β-1,6-吡喃型半乳糖和 β-1,6-吡喃型甘露糖。几个组分中 TM-P2B 抗氧化能力及抗肿瘤能力最强。其氧化自由基吸收能力（ORAC 值）为 2475.33±15.32 μmol Trolox/g。在多糖浓度为 1 mg/mL 时对人肝癌细胞 HepG2 的抑制率达到 90.23±2.17%，是 TM-P2A 的 1.2 倍，TM-P1A 的 3.1 倍。本研究对于松茸的深度开发利用及相关产品的开发具有重要意义。

关键字：松茸；多糖；纯化；抗氧化活性；抗增殖活性

It is well known that polysaccharides play an important role in the development of living organisms. Previous reports have indicated that polysaccharides possess strong bioactivities, such as anti-tumor, immunomodulation, hypoglycemic and antioxidant activities, which have attracted increasing attention in recent years[1]. In addition, important relationships between the bioactivities and structural characteristics of polysaccharides have been demonstrated. For example,

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β-D-glucopyranosyl units have been shown to be very important for immunopotentiating activity, whichis dependent on a helical conformation and on the presence of hydrophilic groups located on the outside surface of the helix[2]. Furthermore, polysaccharides composed of α- or β-glucans or peptide-bound glucans have been shown to exhibit anti-cancer activity in animal models [3].

Tricholoma matsutake, belonging to Subgenus Tricholoma, is a type of ectomycorrhizal symbiotic mushroom. It is one of the most valuable mushroom species worldwide due to its unique flavor and taste as well as several biological activities [4~6]. It has been used not only as food or in food flavoring, but also for the prevention and treatment of diseases in Asian countries, such as China, Japan, and Korea [7]. Polysaccharides extracted from the fruiting body of *T. matsutake* are found to have various bioactivities, such as antioxidative[8], immunoregulation[9], antitumor[10], anti-mutagenic, and hematopoietic activities[2]. It was reported that polysaccharides extracted from *T. matsutake* are mainly composed of glucose, xylose, and galactose [11,12]. Ding et al. isolated a novel heteropolysaccharide from *T. matsutake* and found it has a backbone of β-1,4-glucopyranose with a branch at O-6 composed of α-(1→3)-galactopyranose terminated with α-xylopyranose[11]. Our previous work indicated that the fruiting body of *T. matsutake* contains a large quantity of polysaccharides with strong antioxidant activities[13], but their structure-activity relationships remain unknown. Therefore, it is worthwhile to purify the polysaccharides and characterize their structures.

In the present study, crude polysaccharides were obtained from the fruiting body of *T. matsutake* by ultrasound-assisted extraction. After purification by anion exchange and size-exclusion chromatographies, their structural characteristics were investigated by gas chromatography, high performance gel permeation chromatography, and NMR spectrometry. Moreover, the oxygen radical absorbance capacity (ORAC) and antiproliferative activities of the purified fractions against the human hepatoma cell line HepG2 were evaluated.

1 Materials and methods

1.1 Materials and reagents

Lyophilized *T. matsutake* was obtained from Infinitus Co. Ltd. (Guangzhou, China). The human hepatoma cell line HepG2 was provided by the cell bank of the Shanghai Institute of Cell Biology (Shanghai, China). DMEM medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco/Invitrogen (Grand Island, NY, USA). 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), 2, 2′-azobis (2-methylpropionamidine)-dihydrochloride (AAPH), fluorescein, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dextrans and monosaccharide reference standards (arabinose, rhamnose, fucose, xylose, galactose, glucose and mannose) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DEAE Sepharose Fast Flow and Sephadex G-75 were obtained from GE Healthcare Life Science (Piscataway, NJ, USA). All other chemicals used were analytical grade.

1.2 Separation and purification of *T. matsutake* polysaccharides

The crude *T. matsutake* polysaccharide (TM-P) was prepared according to our previously reported method [13]. As shown in Figure 1A, TM-P was purified sequentially by chromatography with DEAE Sepharose Fast Flow and Sephadex G-75. Briefly, a crude TM-P solution (160 mg/mL, 5 mL) was applied to a DEAE Sepharose Fast Flow column (2.6 × 20 cm) which was sequentially eluted with 400 mL each of distilled water, 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl, with a flow rate of 5 mL/min and collected in 5 mL fractions. The fractions were combined based on chromatographic profiles obtained using the phenol-sulfuric acid method [14]. Three fractions of polysaccharides (TM-P1, TM-P2, and TM-P3) were separately dialyzed against and lyophilized. TM-P1 and TM-P2 were further purified by Sephadex G-75 (1.6 × 100 cm) chromatography, resulting in the fractions TM-P1A and TM-P1B, and TM-P2A and TM-P2B. The four purified fractions were concentrated, dialyzed, and lyophilized for further study. Because TM-P3 exhibited

poor antioxidant activity compared with TM-P1 and TM-P2, it was excluded from the following structural characterization.

1.3 Analysis of monosaccharide compositions

The monosaccharide compositions of polysaccharide fractions were analyzed according to the method of Wang et al. with a slight modification[15]. In brief, polysaccharide samples (5 mg) were hydrolyzed in a sealed glass tube with 4 M trifluoroacetic acid (5 mL) for 2 h at 110 ℃. The acid was evaporated under reduced pressure at 60 ℃, washed with methanol (4 mL) three times and dried. The dried hydrolysates were dissolved in pyridine (2 mL) containing hydroxylammonium (10 mg) and inositol (1 mg, used as internal reference) and the reaction was incubated at 90 ℃ for 30 min. After cooling to room temperature, 2 mL of acetic anhydride was added and the mixture was incubated for another 30 min at 90 ℃. The reaction products were analyzed by gas chromatography/mass spectrometry (GC-MS) (Trace DSQ-II, Thermo Fisher Scientific, Thermo Electron Co., Waltham, MA, USA) with a flame ionization detector and a TR-5MS capillary column (30 m × 0.25 mm × 0.25 μm). The operating conditions for GC-MS were as follows: flow rate of the carrier gas He was 1 mL/min at a split ratio of 10:1; the temperature of the detector and inlet was 250 ℃; the column temperature was increased from 150 ℃ (holding for 2 min) up to 180 ℃ (holding for 2 min) at a rate of 10 ℃/min, then up to 260 ℃ (holding for 5 min) at a rate of 15 ℃/min. Standard monosaccharides (arabinose, rhamnose, fucose, xylose, galactose, glucose, and mannose) were also derivatized and analyzed by GC-MS as references.

1.4 Measurement of molecular weight distribution

The molecular weight distribution of the polysaccharides was measured by high performance gel permeation chromatography (HP-GPC) using a Waters HPLC apparatus (Waters 1525, Waters Co. Ltd., Milford, MA, USA), which was equipped with a TSK-GEL Guard Column (PWXL 6.0 × 40 mm), a TSKGEL4000 K gel column (PWXL 7.8 × 300 mm), and a TSK-GEL2500 K gel column (PWXL 7.8 × 300 mm) (TOSOH Co. Ltd., Tokyo, Japan), with a Waters 2414 Refractive Index Detector. Phosphate buffer (0.2 M, pH 7.0) was used as the mobile phase at a flow rate of 0.6 mL/min. The injection volume was 30 μL and the temperature of the column was maintained at 35 ℃.

1.5 NMR spectroscopy

A total of 30 mg of each polysaccharide fraction was dissolved in 0.5 mL of D_2O. ^1H and ^{13}C NMR 1D and 2D spectra were recorded using a Bruker DRX-600 spectrometer (Bruker, Rheinstetten, Germany) at 25 ℃. Chemical shifts are expressed in ppm. Acetone was used to calibrate the chemical shift of ^{13}C spectra. Tetramethylsilane was used as an internal standard.

1.6 Oxygen radical absorbance capacity (ORAC) assay

The ORAC analyses were performed in 96-well polystyrene black microplates using a Varioskan Flash spectral scan multimode plate reader (Thermo Fisher Scientific). The ORAC values were determined according to the method of Folch-Cano et al. with some modifications as described in our previous report[13,16].

1.7 Antiproliferative activity assays

Antiproliferative activities of the polysaccharides in the human hepatoma cell line HepG2 cell were determined by the MTT-based colorimetric method[17]. Briefly, HepG2 cells were suspended in DMEM medium supplemented with 10% fetal bovine serum, 100 IU/mL of penicillin and streptomycin at 37 ℃ in a humidified atmosphere containing 5% CO_2.

The HepG2 cell suspensions were pipetted into a 96-well plate at a density of approximately 5000 cells/mL (100 μL/well) and cultured for 24 h. Test samples at different concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) were then added separately to each well[18]. After 24-h incubation, MTT reagent (0.5 mg/mL) was added to each well, and the plate was further incubated for 4 h. Then, the media were removed, and 150 μL of DMSO was added to each well for the dissolution of the formazan crystals. The absorbance of each well was read at 490 nm using the Varioskan Flash spectral scan multimode plate reader. The antiproliferative activity wascalculated as a percentage according to the formula:

$$\text{Antiproliferative activity} = \frac{A_c - A_s}{A_c - A_b} \times 100$$

Note: where A_s is the absorbance of sample, A_c is the absorbance of the control (without sample), and A_b is the absorbance of the blank (the absorbance of DMSO).

1.8 Statistical analysis

Data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and expressed as mean ± standard deviation (SD) for at least three replicates. Significance was determined at $p< 0.05$ by analysis of variance (ANOVA) followed by Dunnett's test.

2 Results and discussion

2.1 Extraction and purification of polysaccharides

The crude polysaccharide TM-P was obtained from the fruiting bodies of *T. matsutake* with a yield of 5.76 ± 0.07% (data not shown). Three distinct elution peaks (TM-P1, TM-P2, and TM-P3) from the anion exchange chromatography accounted for 50.35 ± 2.05%, 10.43 ± 1.42%, and 6.52 ± 0.98% of TM-P, respectively[13]. TM-P1 and TM-P2 were further purified by Sephadex G-75 size-exclusion chromatography. As a result, each fraction afforded two elution peaks (Fig. 1B, Fig. 1C, and Table 1), named as TM-P1A (13.8% of TM-P1) and TM-P1B (86.2% of TM-P1), TM-P2A (20.6% of TM-P2) and TM-P2B (79.4% of TM-P2).

2.2 Molecular weight distribution and monosaccharide composition of polysaccharides

High-performance gel permeation chromatography was used to obtain the molecular weight distribution of the polysaccharide fractions. The results showed that the average molecular weights of TM-P1A, TM-P1B, TM-P2A, and TM-P2B were 361.31, 15.63, 372.44, and 4.80 kDa, respectively (Table 1),where the molecular weight of TM-P2B was approximately 1/9 of that of TM-P1A and TM-P2A.

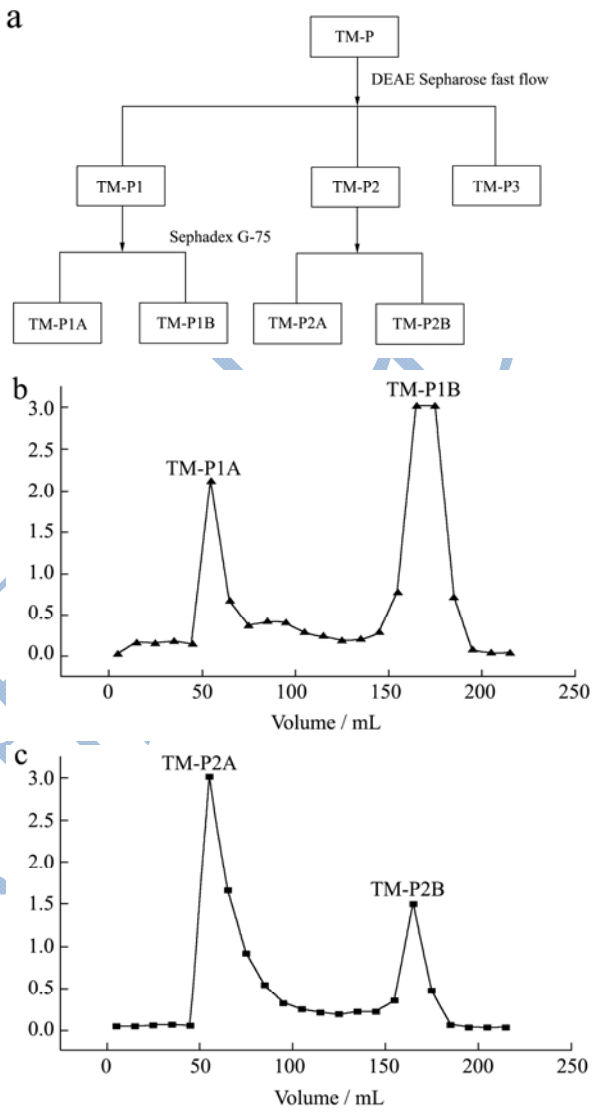

Fig.1 Scheme of purification of crude TM-P (a) and elution curve of polysaccharide fractions (TM-P1 and TM-P2) on size-exclusion chromatography column of Sephadex G-75 (b and c)

Table 1 The compositions, average molecularweights and ORAC values of polysaccharides

	TM-P1A	TM-P1B	TM-P2A	TM-P2B
Composition/%	13.8	86.2	20.6	79.4
Average molecular weight/kDa	361.31	15.63	372.44	4.80
ORAC/(μmol Trolox/g)	211.80±4.68	58.21±1.32	268.66±5.02	2475.33±15.32

As shown in Table 2, TM-P1A and TM-P2B were mainly composed of glucose and galactose, as well as mannose, at molar ratios of 8.7:1.8:1.0 and 8.9:1.3:1.0, respectively. These results indicated that glucose was the predominant monosaccharide, consistent with it forming the backbone of TM-P1A and TM-P2B. Notably, TM-P2A was quite different from TM-P1A and TM-P2B, in that it was composed of glucose, galactose, mannose,

and fucose at a molar ratio of 17.7:7.9:3.9:1.0. Glucose and galactose were the major monosaccharides, consistent with them forming the backbone of TM-P2A. The monosaccharide compositions of *T. matsutake* polysaccharides in this study were slightly different from those previously reports. Ding et al. (2010) which was demonstrated that *T. matsutake* polysaccharides are composed of glucose, galactose, and xylose at a molar ratio of 8:1:1. These differences may be due to the extraction method [19] and/or *T. matsutake* varieties. The degradation of polysaccharides is more extensive with ultrasonic treatment, which may contribute to the differences in monosaccharide composition.

Table 2 The monosaccharide compositions of polysaccharide fractions (molar ratio)

	TM-P1A	TM-P2A	TM-P2B
Glucose	8.7	17.7	8.9
Galactose	1.8	7.9	1.3
Mannose	1	3.9	1
Fucose	nd[a]	1	nd

Note: a Not detectable.

2.3 NMR spectra of polysaccharides

The anomeric proton signal at 5.20 ppm in the ^{1}H NMR spectrum of TM-P1A (Fig. 2a) show that the polysaccharide is linked via glycosidic bonds in the α-configuration. There was no proton signal at 5.40 ppm, indicating that the sugar rings of TM-P1A are pyranose. In the ^{13}C NMR spectrum of TM-P1A (Fig. 3b), the resonances at 92.50~102.20 ppm suggest the presence of both α- and β-anomeric configurations at a ratio of 40:1. This result also confirmed that the sugar residues are mainly linked by α-glycosidic bonds. The anomeric carbon signal at 92.50 ppm was assigned to glucose; however, other anomeric carbon signals were very weak, suggesting a small amount of galactose and mannose. Signals at 67~70 ppm were assigned to 1,6-linked glycosidic bonds. The signal at 69.68 ppm was assigned to C_6 of glucose. There were no signals at 78~85 ppm, indicating that C_2, C_3, and C_4 are un-substituted. These results manifested that TM-P1A contains α-1,6-glucopyranose.

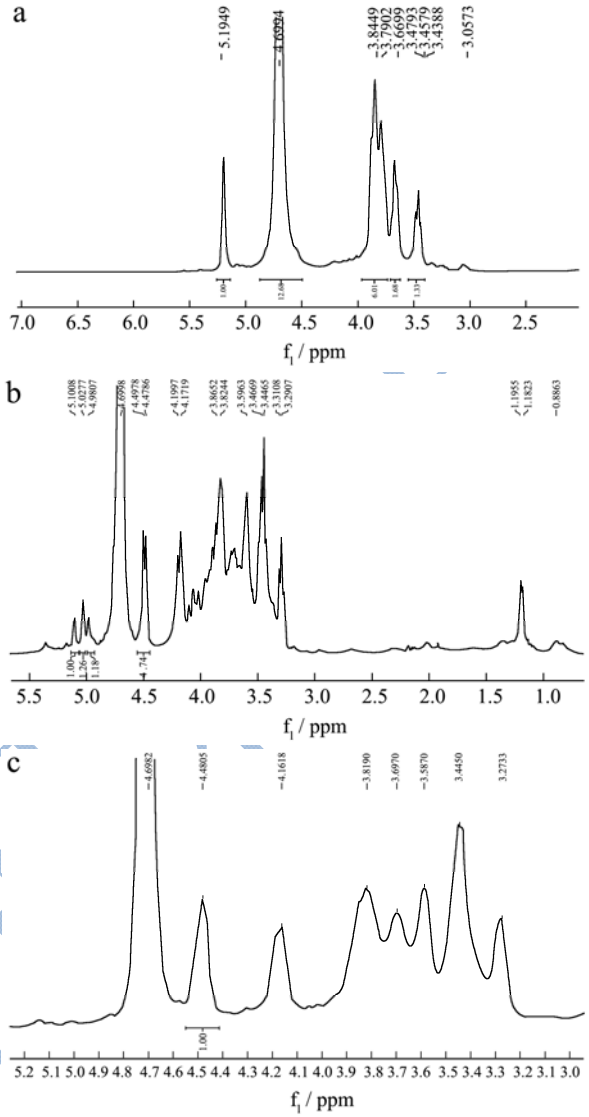

Fig.2 The ^{1}H NMR spectra of the polysaccharide fractions: (a) TM-P1A; (b) TM-P2A; (c) TM-P2B

The anomeric proton signal at 4.48, 4.50, 5.03, and 5.10 ppm in the ^{1}H NMR spectrum of TM-P2A (Fig. 2b) suggests the presence of both α- and β-anomeric configurations at the ratio 0.72:1. The signal at 1.20 ppm was assigned to methyl groups of deoxysugars, suggesting the presence of fucose, which was consistent with the monosaccharide analysis results. There was no proton signal at 5.40 ppm, indicating that the sugar rings of TM-P2A are pyranose. In the ^{13}C NMR spectrum of TM-P2A (Fig. 3b), the resonances at 97.43~102.16 ppm demonstrate the presence of both α- and β-anomeric configurations at a ratio of 0.72:1, which is consistent with the ^{1}H NMR spectrum. Signals at 102.16, 101.68, and 97.43 ppm were assigned to β-glucose, β-galactose, and α-galactose, respectively. Signals at 67~70 ppm were

assigned to 1,6-linked glycosidic bonds. Signals at 60~65 ppm were assigned to un-substituted C_6. There were no signals at 78~85 ppm, indicating that C_2,C_3, andC_4 are un-substituted. The signal at 14.87 ppm was assigned to methyl groups of deoxysugars. These results demonstrate that the backbone of TM-P2A is composed of glucopyranose linked via β-1,6-glycosidic bonds.

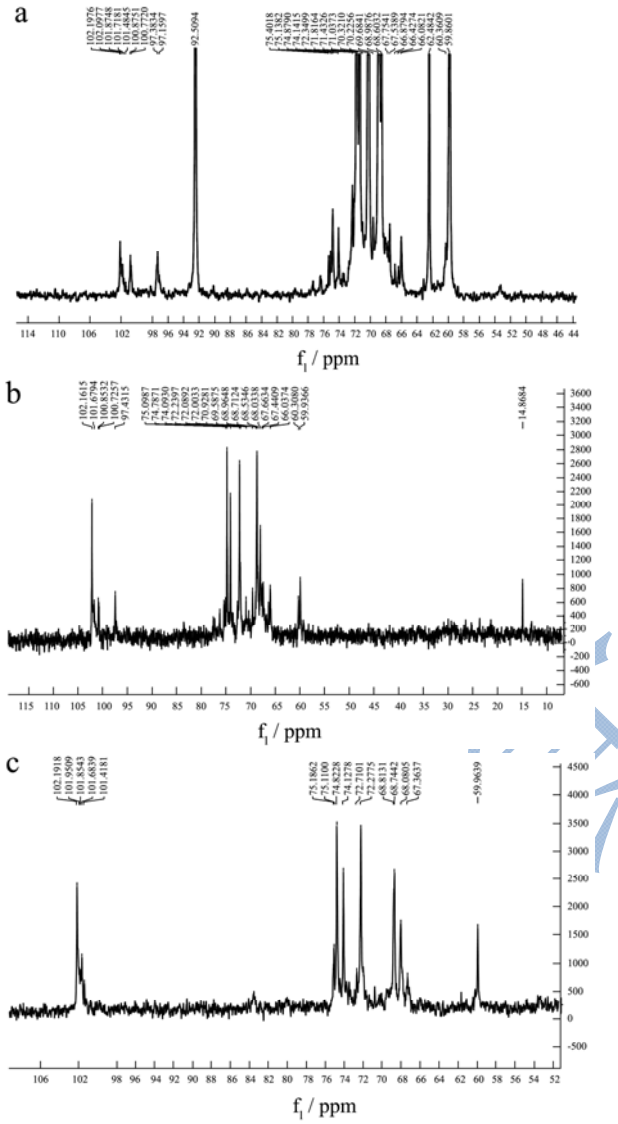

Fig.3 The ^{13}C NMR spectra of the polysaccharide fractions: (a) TM-P1A; (b) TM-P2A; (c) TM-P2B

The anomeric proton signal at 4.48 ppm in the ^{1}H NMR spectrum of TM-P2B (Fig. 2c) shows that the polysaccharide is linked by glycosidic bonds in the β-configuration. As with TM-P1A, the absence of a proton signal at 5.40 ppm indicates that the sugar rings of TM-P2B are pyranose. In the ^{13}C NMR spectrum of TM-P2B (Fig. 3c), the resonances at 101.41, 101.68, and 102.19 ppm suggest that the polysaccharide is linked only via β-glycosidic bonds, which is consistent with the ^{1}H NMR spectrum. Signals at 102.19, 101.68, and 101.41 ppm were assigned to β-glucose, β-galactose, and β-mannose, respectively. Signals at 67~70 ppm were assigned to 1,6-linked glycosidic bonds. An un-substituted C_6 signal was observed at 60.45 ppm. Moreover, no signals were found at 78–85 ppm. These results suggest that TM-P2B is composed of β-glucopyranose, as well as some β-galactopyranose and β-mannopyranose, all of which are linked via 1,6-glycosidic bonds.

2.4 Oxygen radical absorbance capacity (ORAC) of polysaccharides

As shown in Table 1, TM-P2B exhibited the highest ORAC value (2475.33 ± 15.32 μmol Trolox/g), approximately 11.7, 42.5, and 9.2 times that of TM-P1A (211.80 ± 4.68 μmol Trolox/g), TM-P1B (58.21 ± 1.32 μmol Trolox/g), and TM-P2A (268.66 ± 5.02 μmol Trolox/g), respectively. The relative ORAC values of all fractions may be ranked in decreasing order as TM-P2B > TM-P2A > TM-P1A > TM-P1B. The differences in antioxidant activity of the polysaccharide fractions could be the result of differences in their monosaccharide composition, molecular weight, and type of glycosidic bond[20].

2.5 Antiproliferative activities of polysaccharide fractions against HepG2 cells

The antiproliferative activities of polysaccharide fractions TM-P1A, TM-P2A, and TM-P2B on the growth of HepG2 cells were investigated using the MTT assay. As shown in Fig. 4, all polysaccharide fractions showed a dose-dependent effect within the concentration ranges tested. All fractions significantly inhibited the growth of HepG2 cells (p< 0.05). TM-P2B showed the strongest antiproliferative activity among three fractions. At 1.0 mg/mL, the antiproliferative activity of TM-P2B was 90.23 ± 2.17%, 1.2 and 3.1 times than those of TM-P2A and TM-P1A, respectively. The relative growth-inhibiting activities of the three fractions were observed to be in the decreasing order of TM-P2B > TM-P2A > TM-P1A, similarly to the relative rankings of ORAC values, indicating that the antitumor activities of the

polysaccharides are correlated with their antioxidant activities.

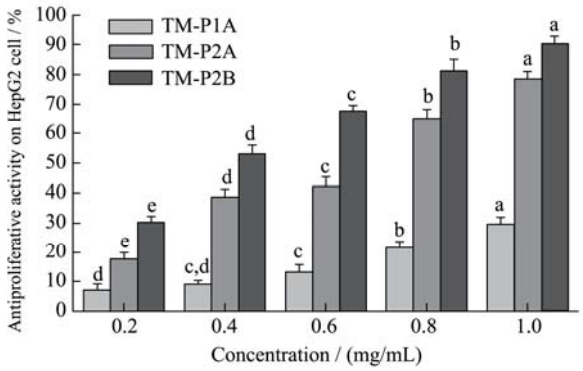

Fig.4 The anti-proferative activities of polysaccharides fractions on the growth of HepG2 cells

Note: The data with different lowercase letters are significantly ($P<0.05$) different for the same fraction at different concentrations

It has been reported that the bioactivities of polysaccharides are dependent on their molecular weight, chemical composition, structure of the polymeric backbone, and degree of branching [21,22]. It has been reported that most bioactive mushroom polysaccharides are β-(1→3) and (1→6)-glucans; some of these were shown to be heteropolysaccharides[23]. Gonzaga et al. found that an α-(1→4)-β-(1→6)-glucan complex of *Agaricus blazei* Murrill mushroom had strong anti-tumor effects *in vivo* but produced no cytotoxicity in tumor cells*in vitro*[24]. In our study, TM-P2B and TM-P2A (both primarily with β-glucans), which were shown to have stronger antioxidant and antitumor activities than those of TM-P1A (mainly with α-glucans), may further confirm that polysaccharides composed of β-linked monosaccharides have more potent bioactivities than those composed of α-linkages[25].

3 Conclusions

In the present study, polysaccharides of *T. matsutake* (TM-P) were purified, resulting in four distinct fractions: TM-P1A, TM-P1B, TM-P2A, and TM-P2B. The results demonstrated that TM-P2B showed the most robust antioxidant and antiproliferative activities among all the fractions. The differences in their bioactivities were likely due to differences in their molecular weight distribution, monosaccharide composition, and glycosidic bond. TM-P2B and TM-P2A (both mainly with β-glucans) had more potent antioxidant and antitumor activities than TM-P1A (mainly with α-glucans), further confirming that polysaccharides composed of β-glycosidic linkages have more potent bioactivities than those composed of α-linkages. Further investigations into their structure-activity relationships are in progress.

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