



Structure characterization of polysaccharide isolated from the fruiting bodies of *Tricholoma matsutake*

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ABSTRACT

A novel heteropolysaccharide was isolated from the fruiting bodies of *Tricholoma matsutake* through DEAE–cellulose column and Sephadex G-100 column. The *T. matsutake* polysaccharide (TMP-A) had a molecular weight of 8.89×10^4 Da and was mainly composed of 4-D-Glc:4,6-D-Glc:3-D-Gal:T-D-Xyl and with the ratio of 7:1:1:1. The structural features of TMP-A were investigated by a combination of total hydrolysis, methylation analysis, gas chromatography–mass spectrometry (GC–MS), scanning electron microscope (SEM), infrared (IR) spectra, nuclear magnetic resonance (NMR) spectroscopy and dynamical analysis of the atomic force microscope (AFM) studies. The results indicated that TMP-A had a backbone of 1,4-β-glucopyranose which branches at O-6 composed of an (1 → 3)-α-galactopyranose residue and terminated with α-xylopyranose residue. Chain conformation study showed that the polysaccharide took random coil compact conformation.

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1. Introduction

Oxidation is essential to many organisms for the production of energy to fuel biological processes. However, the uncontrolled production of superoxide anion free radicals is involved in onset of many diseases such as cancer, atherosclerosis and degenerative processes (Mau, Tsai, Tseng, & Huang, 2005). Plant polysaccharides have shown strong antioxidant properties and can be used as a novel potential antioxidant (Hu, Xu, & Hu, 2003; Ramarathnam, Osawa, Ochi, & Kawaishi, 1995; Wang & Luo, 2007). In addition, polysaccharides extracted from mushrooms, such as *Lentinus edodes*, *Ganoderma tsugae* and *Cordyceps sinensis*, have also exhibited antioxidant properties by their free radical scavenging ability (Chen, Xie, Nie, Li, & Wang, 2008; Chen, Zhong, Zeng, & Ge, 2008; Dong & Yao, 2008; Tseng, Yang, & Mau, 2008; Yu, Yin, Yang, & Liu, 2008). Such natural antioxidants may be effective to protect humans from free radicals damage and many chronic diseases (Kinsella, Frankel, German, & Kanner, 1993).

Tricholoma matsutake is a kind of fungi belonging to Subgenus *Tricholoma*. As a traditional edible fungus in oriental countries, it

has been used for the prevention and treatment of diseases for several thousand years (Gong, Su, Chen, Feng, & Cao, 2002; Gurein et al., 2003; Hur, Park, Kang, & Joo, 2004). As an extract from the fruiting bodies of *T. matsutake*, polysaccharides (TMP) have shown strong anti-tumor bioactive properties (Gao, 1997; Liu, 2001; Mikio et al., 1999; Shung, 2004). The determination of the structure of polysaccharides is necessary to establish a fundamental guide for assessing the bioactivity and pharmacological mechanism in vivo/in vitro.

In this work, we reported a novel water-soluble polysaccharide was extracted and purified from the fruiting bodies of *T. matsutake* using a DEAE–cellulose column chromatography and a Sephadex G-100 column chromatography. Its chemical structures were further characterized and our results provided a basis for future pharmacological studies.

2. Materials and methods

2.1. Chemicals

The fruiting bodies of *T. matsutake* were collected in Xiaojing country of Sichuan province, China, and were authenticated by Prof Sao-rong Ge (College of Life Sciences, Sichuan University, Chengdu, China). At the same time, a voucher specimen had been preserved in Key Laboratory for Biological Resource and Ecological Environment of Education Ministry, College of Life Sciences, Sichuan University. DEAE–Cellulose 52 and Sephadex G-100 were purchased from Sigma–Aldrich (Mainland, China). Monosaccharide standards, Dex-

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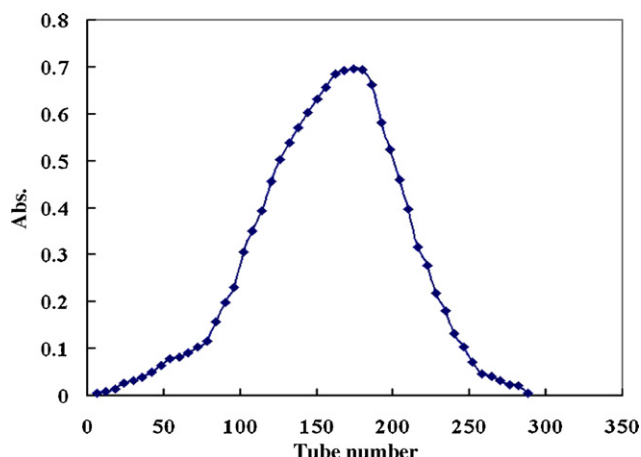


Fig. 1. Elution curve of TMP-A in Sephadex G-100 column chromatography.

tran T-500, T-110, T-70, T-40, and T-10, were purchased from Beijing Biodee Biotechnology Co., Ltd. (Beijing, China). All other reagents used were of analytical grade.

2.2. Extraction of polysaccharides from *T. matsutake*

The fruiting bodies (200 g) of *T. matsutake* were soaked with 95% EtOH for 6 h to remove lipids by filtration. Then the residue was dried and extracted with boiling water for three times (6 h for each). The filtrate was concentrated, dialyzed (MWCO 5000, Sigma), and centrifuged to remove insoluble material and small molecular compounds. Then the supernatant was added with 3 volumes of 95% EtOH to precipitate crude polysaccharides. The precipitate was recovered by centrifugation and washed successively with absolute EtOH, followed by drying in vacuo at 45 °C, yielding the crude *T. matsutake* polysaccharide (TMP) (32.8 g, recovery 16.4%). Sevag method (Staub, 1965) was used for the deproteination of TMP, 30% H₂O₂ for decoloration, respectively.

2.3. Purity and fractionation of polysaccharides

The resulting polysaccharide solution was dialyzed, concentrated and lyophilized. TMP (8 g) was subjected to a DEAE-cellulose column (Tris-HCl, pH 7.0, 4.5 cm × 50 cm, Cl[−]) and eluted stepwise with 0, 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0 M NaCl. The eluate was monitored by the phenol-sulfuric acid method (Dubois, Gillis, Hamilton, Rebers, & Smith, 1956). The 0 M NaCl elution was concentrated, dialyzed, and lyophilized. Then the resulting polysaccharide was purified on a Sephadex G-100 column (2.6 cm × 60 cm) and eluted with 0 M NaCl. The resulting *T. matsutake* polysaccharide, named TMP-A, was obtained by the above processes and the yield rate of TMP-A was 0.22% (0.432 g) for the starting material.

2.4. Measurement of molecular weight of TMP-A

High performance gel permeation chromatography (HPGPC) was carried out to measure molecular weight. The column was calibrated with standard T-series Dextran (T-500, T-110, T-70, T-40 and T-10). The data were processed with Waters GPC (Millennium32 software).

2.5. Monosaccharide composition analysis

The polysaccharide TMP-A (5.0 mg) was hydrolyzed with 2 M trifluoroacetic acid at 110 °C for 6 h on the mechanism of acid-catalyzed hydrolysis (Nelson & Cox, 2004; Yu et al., 2009). Excess acid was removed by co-distillation with Methanol after the hydrolysis was completed. One part of the hydrolysate (1.0 mg) was used for thin layer chromatography analysis as described previously (Liu, Dong, & Fang, 2001; Partridge, 1949; Zhang, 1999), and the other (1.0 mg) was dissolved in pyridine (0.2 mL). The derivatization reaction was initiated by addition of hexamethyl-disilazane (0.2 mL) and trimethyl chloro-silicane (0.2 mL) according to the method described by Dong, Zhang, Lin, and Fang (1995) and Guentas et al. (2001). The resulting supernatant was examined by GC-MS at a temperature program of 50–230 °C with a rate of 2 °C/min (Chen, Xie, et al., 2008; Chen, Zhong, et al., 2008; Needs & Selvendran, 1993).

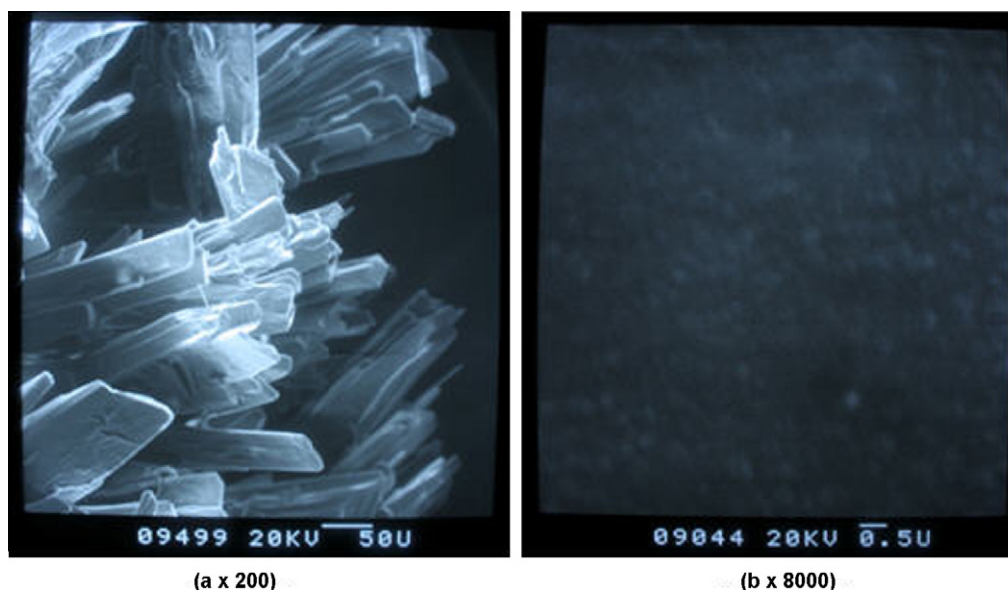


Fig. 2. The purified polysaccharide TMP-A were imaged by SEM.

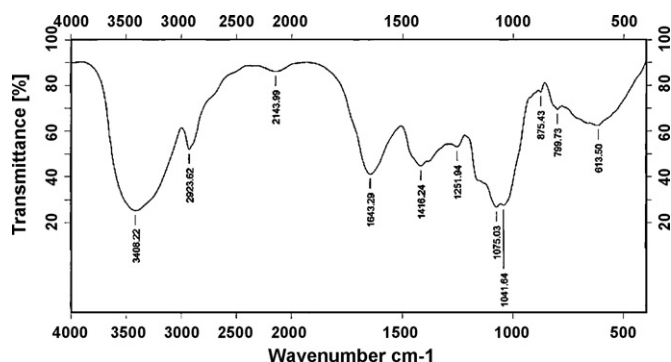


Fig. 3. FTIR spectra of polysaccharide TMP-A.

2.6. Methylation analysis

The polysaccharide, TMP-A (10 mg), was methylated according to the Hakomori method (Hakomori, 1964). After being methylated completely, the permethylated polysaccharide was depolymerized with 90% aqueous formic acid (3 mL) for 10 h at 100 °C in a sealed tube. The methylated sugars were derivatized using the method described by Dong et al. (1995) and analyzed by GC–MS.

2.7. UV and infrared (IR) spectra analysis

TMP-A was tested in UV from 200 nm to 600 nm. And Infrared analysis of the samples was obtained by grinding a mixture of polysaccharide with dry KBr and then pressing in a mold. Spectra were run in the 4000–400 cm^{-1} region (Kumar, Joo, Choi, Koo, & Chang, 2004).

2.8. Nuclear magnetic resonance (NMR) experiment

^1H NMR spectra and ^{13}C NMR spectra were recorded on a Varian Unity INOVA 400/45 in D_2O with tetramethylsilane as internal standard.

2.9. Atomic force micrograph (AFM)

The normal sample preparation procedure consisted of spreading of a dilute (25 $\mu\text{g/mL}$) polymer solution onto a freshly cleaved mica surface and successive air-drying under ambient pressure, temperature, and humidity (Kirby, Gunning, & Morris, 1995; Michela, Meredith, Paola, Neil, & Rizzo, 2009). The atomic force microscopy was operated in the tapping-mode (Gunning et al., 2003; Morri, Gunning, Kirby, Round, & Waldron, 1997).

3. Results and discussion

3.1. Extraction, purity and composition of polysaccharides

The crude polysaccharide, named TMP, was obtained from the fruiting bodies of *T. matsutake* with a yield of 16.4%. After fractionation on DEAE-Cellulose 52 and Sephadex G-100 column chromatography, 432 mg of TMP-A was obtained from the 0 M NaCl eluate and detected by the phenol–sulfuric acid assay (Dubois et al., 1956). The homogeneity of the polysaccharide was elucidated by the following tests: TMP-A was eluted from gel-filtration chromatography on Sephadex G-100 column and was also detected by the phenol–sulfuric acid assay as a single peak (Fig. 1). The amber-coloured crystal TMP-A were shown by SEM in Fig. 2. No absorption at 280 nm and 260 nm in UV absorption spectra of TMP-A demonstrated the absence of protein and nucleic acid in this polysaccharide and it had the same optical rotation: $[\alpha]_{\text{D}}^{20} - 1.648^\circ$ (c0.5, water), in different low concentration of ethanol using HK7-SGW-1 automatic optical polarimeter at room temperature. Weight-average molecular weight was around 8.89×10^4 Da. The Rf values of hydrolyzed sample monosaccharide using TLC were 0.272, 0.538, 0.156, respectively, which were the same as the standard monosaccharide (Glucose, Galactose, Xylose). The color of monosaccharides (Glc, Gal) were shown clearly as blue and Xyl as brown-green. The three monosaccharides, D-Glc, D-Xyl and D-Gal were also identified using the hydrolysate of TMP-A by GC–MS and their ratios were 8:1:1. TMP-A was supposed to contain the D-configuration monosaccharide according to GC–MS analysis.

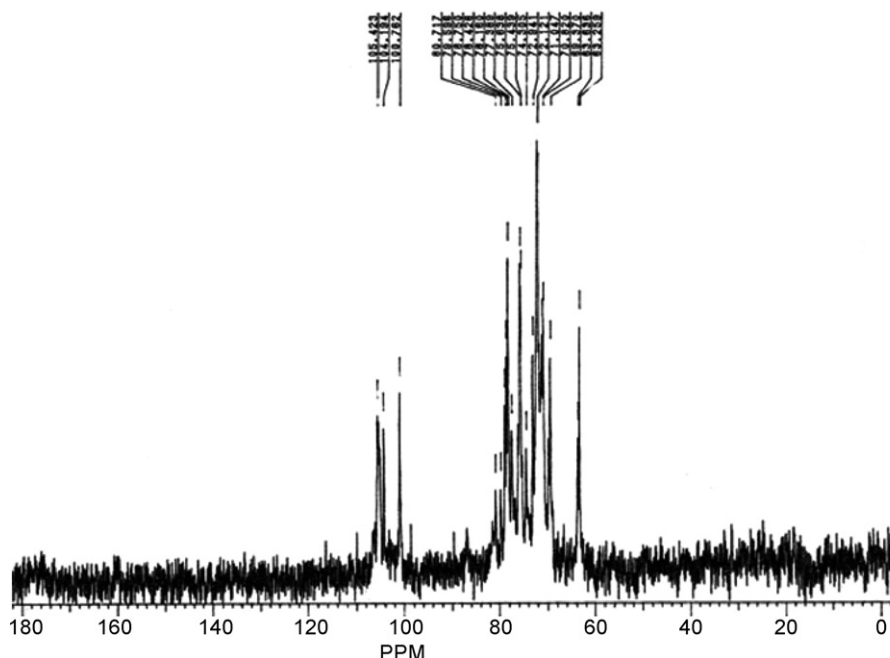


Fig. 4. The ^{13}C NMR spectra of polysaccharide TMP-A.

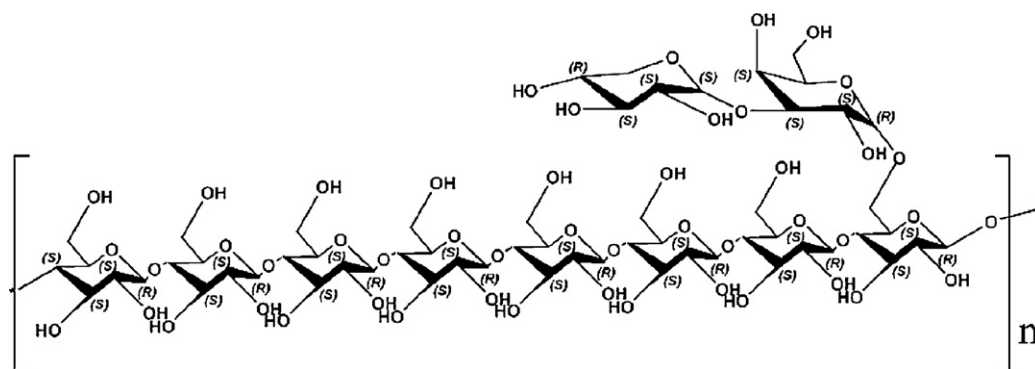


Fig. 5. Predicted chemical structure of polysaccharide TMP-A.

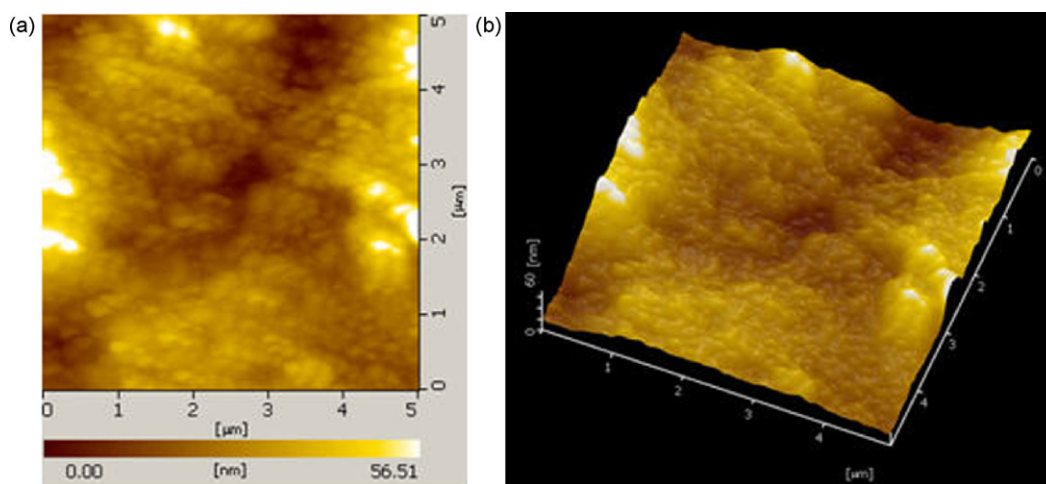


Fig. 6. Atomic force microscopy (AFM) planar (a) and cubic images (b) of molecular structure of TMP-A from the fruiting bodies of *Tricholoma matsutake*.

3.2. Structure elucidation of TMP-A

The intensity of bands around 3408.22 cm^{-1} in the IR spectrum (Fig. 3) was due to the hydroxyl stretching vibration of the polysaccharide and as expected they were broad. The bands in the region of 2923.62 cm^{-1} were due to C–H stretching vibration and the bands in the region of 1643.29 cm^{-1} were due to associated water (Cao et al., 2006; Park, 1971). Two strong absorption bands at 1075.03 cm^{-1} , 1041.64 cm^{-1} in the range of $1200\text{--}1000\text{ cm}^{-1}$ suggested that the monosaccharide of TMP-A had a pyranose ring (Barker, Bourne, Stacey, & Whiffen, 1954; Cui et al., 2008) (Fig. 3). The absorption at 875.43 cm^{-1} indicated that TMP-A had β -glycopyranosidic linkages, which was also indicated by the anomeric proton signals at $\delta\ 4.660$ in the ^1H NMR (400 MHz) (Chakraborty, Mondal, Pramanik, Rout, & Islam, 2004; Kim et al., 2000). Moreover, the characteristic absorptions at 799.73 cm^{-1} indicated α -configurations existing in the polysaccharide (Wu & Tu, 2005), which was in good agreement with the anomeric proton signals at $\delta 5.263$, $\delta 5.182$ and $\delta 5.107$ in the ^1H NMR (400 MHz) spectrum. The resonances in the region of $98\text{--}106\text{ ppm}$ in the ^{13}C NMR (200 MHz) spectrum of TMP-A were attributed to the anomeric carbon atoms of glucopyranose (GlcP), galactopyranose (GalP) and xylopyranose (XylP) (Wang, Liang, & Zhang, 2001; Wang, Luo, & Liang, 2004; Zhao, Kan, Li, & Chen, 2005). In the anomeric carbon region, signals at $\delta 105.2$ could be attributed to C-1 of $\rightarrow 4\text{--}\beta\text{-D-GlcP-(1}\rightarrow\text{)}$; $\delta 105.4$ to C-1 of $\rightarrow 4,6\text{--}\beta\text{-D-GlcP-(1}\rightarrow\text{)}$; $\delta 100.7$ to C-1 of $\rightarrow 3\text{--}\alpha\text{-D-GalP-(1}\rightarrow\text{)}$; $\delta 104.1$ to C-1 of $\alpha\text{-D-XylP-(1}\rightarrow\text{)}$, respectively (Fig. 4). All the assignment of the carbon atoms signals was shown in Table 1.

After methylation by the modified method (Hakomori, 1964) for four times, the methylated polysaccharide was depolymerized and converted into partially methylated ramifications. The analysis of the methylated monosaccharide was conducted by GC–MS. The information in MS showed that fragment ion peaks were consistent with data of D-configuration monosaccharide fragment ions peaks which can be concluded that the glucose, galactose and xylose residues were D configuration. Methylation analysis for TMP-A proved that the glucose residues were 2,3-bis-substituted and 2,3,6-tri-substituted, respectively, the galactose residues were 2,4,6-tri-substituted, and the xylose residue was 2,3,4-tri-substituted (see Table 2 and Fig. 5). Both results of monosaccharide composition analysis and methylated linkage analysis of TMP-A indicated that $(1\rightarrow 4)$ -linked-glucose was the largest amounts residue of the polysaccharide structure, and the branched residue was $(1\rightarrow 4,6)$ -linked-glucose revealing that $(1\rightarrow 4)$ -linked-glucose should be possible to form the backbone structure. The relative amounts of $(1\rightarrow 4,6)$ -linked-glucose indicated that approximate branch ratios could theoretically be 12.5%,

Table 1
 ^{13}C NMR chemical shift data (δ , ppm) for polysaccharide TMP-A.

Sugar residues	Chemical shifts, δ (ppm)					
	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆
$\rightarrow 4\text{--}\beta\text{-D-GlcP-(1}\rightarrow\text{)}$	105.239	69.370	78.160	72.941	78.750	63.636
$\rightarrow 4,6\text{--}\beta\text{-D-GlcP-(1}\rightarrow\text{)}$	105.423	69.370	78.426	72.121	79.598	63.636
$\rightarrow 3\text{--}\alpha\text{-D-GalP-(1}\rightarrow\text{)}$	100.762	70.820	77.388	75.439	80.717	63.258
$\alpha\text{-D-XylP-(1}\rightarrow\text{)}$	104.194	71.047	75.638	74.305	72.121	

Table 2

GC–MS results of methylation analysis of TMP-A.

Methylated sugar	Linkage	Mass fragments (<i>m/z</i>) (relative abundance, %)
2,3,6-Me ₃ -Glc	1,4–	45, 59, 73, 88, 101, 116, 133, 146, 159, 232 (100, 62, 53, 38, 21, 14, 20, 3, 7, 2.3)
2,3-Me ₂ -Glc	1,4,6–	45, 59, 73, 88, 101, 116, 133, 146, 174, 232 (100, 56, 43, 36, 23, 15, 20, 4, 5, 2.4)
2,4,6-Me ₃ -Gal	1,3–	45, 59, 73, 89, 116, 146, 159, 191, 204, 233 (100, 63, 46, 29, 19, 2.7, 3, 4.3, 2.1, 1.7)
2,3,4-Me ₃ -Xyl	T–	45, 59, 73, 88, 101, 116, 133, 146, 174 (100, 47, 35, 31, 29, 18, 12, 4.7, 1.9)

namely on average one branching point for each eight residues of backbone. Residues of branch structure were (1 → 3)-linked-galactose and terminated with α-xylopyranose residue, which also get from those data of monosaccharide composition analysis and methylated linkage analysis. It is concluded that a repeating unit of TMP-A has a backbone of (1 → 4)-β-D-glucose residues which branches at O-6 based and the branch was supposed to be the composition of an (1 → 3)-α-D-galactose residue and one terminated with α-D-xylose residue. The predicted structure of the novel polysaccharide TMP-A was shown in Fig. 5.

The topographical AFM planar image of TMP-A deposited from a 25 μg/mL water solution was shown in Fig. 6a. Only spherical lumps can be seen which was in good agreement with the measurement performed by SEM (Fig. 2b) and the diameter and height of the lumps ranged from 20 nm to 56 nm (Fig. 6a). The heights of the spherical structures were much higher than that of a single polysaccharide chain (about 0.1–1 nm), suggesting that molecular aggregation was involved (Fig. 6b). We can see that irregularly shaped large structures were formed together like worm from AFM cubic image of TMP-A (Fig. 6b), which suggested that TMP-A could take random coil compact conformation. The conformation might be related to its side chains (Marit, Gjertrud, Pawel, Berit, & Bjørn, 2003; Wang et al., 2010).

4. Conclusions

According to the results above, it was concluded that the highly purified novel polysaccharide obtained from *T. matsutake* is a heteropolysaccharide namely TMP-A. The present study also showed that TMP-A consisted of three monosaccharides, namely D-Glc, D-Xyl and D-Gal and their ratios were 8:1:1 by GC–MS. Structure study demonstrated that TMP-A had a backbone of (1 → 4)-β-glucopyranose residues which branches at O-6 and the branches were composed of an (1 → 3)-α-galactopyranose residue and terminated with α-xylopyranose residue. Chain conformation study showed that the polysaccharide took random coil compact conformation. Further work is necessary to reveal detailed pharmacological effects of TMP-A including antioxidant, anticancer properties and degenerative processes with aging, etc.

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