Isolation and structural elucidation of a novel homogenous polysaccharide from *Tricholoma matsutake*

Hua Cheng, Yan Jia, Ling Wang, Xiaoying Liu, Guangrong Liu, Li Li and Congfen He

"Beijing Key Laboratory of Plants Resource Research and Development, School of Science, Beijing Technology and Business University, Beijing 100048, P.R. China; "Infinitus International (China) Company, Ltd., Guangzhou, Guangdong 510665, P.R. China

(Received 16 December 2014; final version received 13 March 2015)

A crude polysaccharide possessing antitumour, radiation-resistant and anti-ageing attributes was extracted from *Tricholoma matsutake* by water extraction and alcohol precipitation. From this crude polysaccharide, a homogeneous polysaccharide, TMP-5II, was successfully purified by Sephacryl S-300 column chromatography. The average molecular weight ($M_w$) of TMP-5II was 15.76 kDa. Monosaccharide analysis indicated that the homogeneous polysaccharide contained four different residues: D-glucose, D-galactose, D-mannose and D-fucose. Attenuated total reflectance infrared spectroscopy revealed characteristics typical of carbohydrate polymers and a peak typical of a $\beta$-type glycosidic bond. TMP-5II was selected for structural characterisation by nuclear magnetic resonance (NMR) analysis. According to $^1$H NMR, $^{13}$C NMR and two-dimensional-NMR analysis, TMP-5II contains two kinds of linkages, $\beta$ and $\alpha$, at a ratio of 4:1. Preliminary results indicated that the polysaccharide had (1-4)-beta-pyran glucose as the main chain, and a branched chain in the O-6 location with fucose (1-2) mannose (1-3)-alpha-pyran galactose.

**Keywords:** polysaccharide; *Tricholoma matsutake*; GC-MS; 2D-NMR; structure elucidation

1. Introduction

*Tricholoma matsutake* is a fungus belonging to the subgenus *Tricholoma*. As a traditional edible fungus in East Asia, it has been used for several thousand years in the prevention and treatment of diseases, such as diabetes and cardiovascular diseases (Gong et al. 2002). Polysaccharide is rich in *T. matsutake*, with multiple bioactivities. It can enhance immunity and has antitumour effects in humans, as well as several other bioactivities, such as anti-oxidant (Ebina et al. 2002;
Mau et al. 2002; Kim et al. 2008). An extract from its fruiting bodies, *T. matsutake* polysaccharides (TMPs), has shown strong antitumour bioactivity (Liao 2010). Although TMPs have many important bioactivities, the theory about the structure of *T. matsutake* polysaccharides is rarely reported in present study works. Therefore, it is necessary to determine the structure. The determination of polysaccharide structure is also helpful to evaluate the pharmacological potential of polysaccharides and to study the structure-activity relationship at the molecular level.

Here, we report that a novel water-soluble polysaccharide was extracted and purified from the fruiting bodies of *T. matsutake* using a Sephacryl S-300 chromatography column. In this study, a crude polysaccharide was extracted by water decoction and alcohol precipitation, and subsequently purified into nine fractions (TMP-1-9) using graded alcohol precipitation. The purified TMP-5 was further applied to a Sephacryl S-300 column to obtain sub-fractions, until a homogenous polysaccharide was obtained. The molecular weight and monosaccharide composition of the homogenous polysaccharide were analysed. Furthermore, the representative polysaccharide TMP-5II was selected for determination of its polymer chain structures using attenuated total reflectance (ATR) infrared spectroscopy, one-dimensional (1D)-nuclear magnetic resonance (NMR) and two-dimensional (2D)-NMR. The chemical structures of TMP-5II were further characterised, providing a basis for future pharmacological studies.

2. Results and discussion

2.1 Preparation and basic properties

After extraction in boiling water, decolorisation by activated carbon adsorption and filtration with infusorial earth, the extracted solution was obtained. Through alcohol precipitation, the crude polysaccharide was obtained from *T. matsutake* as described previously. The yield of the crude polysaccharide was 3.63%, and the polysaccharide content in the dried crude polysaccharide was 43.4% detected by phenol sulphuric acid method. The purity of TMP-5 detected by gel permeation chromatography-multi-angle light scattering (GPC-MALLS) was 63.1%. After further Sephacryl S-300 chromatography, the TMP-5 product yielded two sub-fractions, TMP-5I and TMP-5II. Both of the sub-fractions were eluted as a single symmetric peak (Figure S1). The yields of the two fractions were 11.3% and 52.8%, respectively. It was also shown that polysaccharide purities of the two fractions were both over 95%. TMP-5II was selected for further structural investigation.

2.2 Purity and molecular weight analysis

TMP-5II was collected in tubes 29–36 of the elution curve. TMP-5II was determined to be a single symmetric peak on the GPC-MALLS system (Figure S2), indicating that they are both homogeneous polysaccharides. The molecular weight (*M*<sub>w</sub>) of TMP-5II, with dn/dc 0.147 mL/g, was also determined using GPC-MALLS. The average molecular weight of the purified polysaccharide TMP-5II was 15.76 kDa, with an uncertainty of 2% and a polydispersity index of 1.05. The retention time on the Sephacryl S-300 column was 27.5 min.

2.3 Monosaccharide composition

Seven monosaccharides were analysed by gas chromatography–mass spectrometry (GC–MS) after being acetylated (Figure S3; Table S1).

After TMP-5II was hydrolysed and acetylated, its monosaccharide composition was analysed by GC-MS (Figure S4; Table S2). Four peaks appeared in the chromatograph for TMP-5II. The peaks of all monosaccharide derivatives from TMP-5II were sharp and symmetrical.
Furthermore, comparison of the monosaccharide peaks of this sub-fraction with standard monosaccharides indicated that it contained four monosaccharide residues: D-glucose, D-mannose, D-fucose and D-galactose (Xiong et al. 2009).

2.4 Infrared (IR) spectroscopy analysis
IR spectroscopy was used in this study to provide additional structural information on the polysaccharide TMP-5II (Figure S5). The different absorption bands observed in the IR analysis were assigned as described previously in the literature. For the sample, a broad band centred at 3358 cm\(^{-1}\) was assigned to hydrogen-bonded hydroxyl groups. An intense band centred at 2911 cm\(^{-1}\) was due to the \(\text{ZCH}\) stretching and showed characteristic absorption for polysaccharides. The absorption band centred at 1631 cm\(^{-1}\) was caused by \(\text{OH}\) flexural vibrations within the polysaccharide. The group of bands that extended from 1480 to 1350 cm\(^{-1}\) was assigned to \(\text{CH} (\text{O-CH}_2)\) flexural vibrations. The intense band extending between 1200 and 1020 cm\(^{-1}\) corresponded to C–O stretching vibrations. The absorption band centred at 920 cm\(^{-1}\) was caused by the C–O (O–CH\(_2\)) stretching vibrations. The absorption band centred at 873 cm\(^{-1}\) was associated with the \(\beta\)-type glycosidic bond. Moreover, the characteristic absorptions at 798 cm\(^{-1}\) indicated \(\alpha\)-configurations within the polysaccharide. All the absorption bands listed above are characteristic IR peaks of carbohydrate polymers and primarily indicated the polysaccharide nature of TMP-5II.

2.5 \(^1\text{H}\) NMR and \(^{13}\text{C}\) NMR
The polysaccharide fraction TMP-5II was analysed by \(^1\text{H}\) NMR and \(^{13}\text{C}\) NMR spectroscopy (Figure S6 and S7, respectively). Three obvious chemical shifts of anomeric protons were found at \(\delta\)5.203, \(\delta\)5.129 and \(\delta\)5.079 ppm in the \(^1\text{H}\) NMR spectrum, indicating that the polysaccharide contained three residues (named A, B and C). However, according to the monosaccharide analysis, four residues were expected. This suggests that there exists some structural feature that is masking the signal from one residue. The molar proportions of residues A, B and C were 1.42, 1.81 and 1.67, respectively, when estimated by the ratio of peak area of the integration of the H-1 signal. The corresponding \(\text{H}\) chemical shifts of residues A, B and C at other positions were packed in the range of \(\delta\)3.3–4.3 ppm and were difficult to discriminate. In addition, \(\delta\)1.296 is the \(\text{H}\) signal of the \(\text{CH}_3\) residue of D-fucose. Similar results to those determined here have been observed in previous studies (Snyder et al. 2006; Dai et al. 2009).

Based on component analysis, IR analysis and data from the literature (Chattopadhyay et al. 2007; Ge et al. 2009; Wu et al. 2009), the \(^{13}\text{C}\) NMR chemical shifts in the area of anomeric carbon atoms also suggested two types of linkage being present in TMP-5II. The C-1 signal for each residue was detectable at \(\delta\)103.073, \(\delta\)101.772, \(\delta\)101.665 and \(\delta\)98.368 ppm. A signal at \(\delta\)15.783 was the C signal of the \(\text{CH}_3\) residue of D-fucose. Three signals were expected between \(\delta\)62 and \(\delta\)60 ppm; however, only two were present. Those peaks (\(\delta\)60.847 and \(\delta\)61.227 ppm) were associated with C-6 of the residues, thus indicating that there is a \(\beta\)-linkage. The shifts in the region \(\delta\)75 to \(\delta\)79 ppm could be assigned to a substituted carbon atom. Owing to the complexity of the \(^{13}\text{C}\) NMR spectrum of TMP-5II, it was difficult to assign the remaining peaks, which were more or less broad, to the respective residues. The absence of signals in the \(\delta\)88 to \(\delta\)90 ppm range demonstrated that TMP-5II has a long backbone and a short-branched chain.

2.6 2D-NMR analysis
The assignment of \(^1\text{H}\) and \(^{13}\text{C}\) resonances and the linkages of residues were obtained from \(^1\text{H}–^{13}\text{C}\) 2D correlation NMR experiments (Snyder et al. 2006; Ma et al. 2008). The single-bond
correlations between the protons and the corresponding carbons obtained from heteronuclear single-quantum coherence (HSQC) spectra (Figure S8) of TMP-5II in D$_2$O enabled all the $^{13}$C to be assigned. The indirect-bond correlations between the protons and the corresponding carbons obtained from heteronuclear multiple bond coherence (HMBC) spectra (Figure S9) of TMP-5II in D$_2$O enabled all the $^{13}$C to be assigned.

Through a series of methods and experiments, many qualitative and quantitative informations of TMP-5II were obtained during the study step by step, such as molecular weight, composition species and proportions of monosaccharide, functional groups and chemical bonds, as well as chemical environment of $^1$H NMR and $^{13}$C. Furthermore, according to the C and H signals in 2D NMR, the preliminary hypothesis was that the polysaccharide had (1-4)-beta-pyran glucose as its main chain and a branched chain in the O-6 location with fucose (1-2) mannose (1-3)-alpha-pyran galactose.

3. Experimental

3.1 Materials and reagents

The fruiting bodies of T. matsutake were harvested in Yunnan Province, China. A voucher specimen (TM201001) is deposited in the Herbarium of the Institute of Medicinal Plant Development, Chinese Academy of Medical Science and Peking Union Medical College.

Sephacryl S-300 was purchased from American Sigma Company (Beijing, China), as were seven standard monosaccharides (D-xylose, D-arabinose, D-mannose, D-glucose, D-galactose, D-fucose and L-rhamnose).

3.2 Extraction of polysaccharides from T. matsutake

The fruiting body powder (100 g) of T. matsutake was extracted twice by decoction. The extracted solution was decolourised with activated carbon and then filtered with infusorial earth and centrifuged. The supernatant was concentrated and then precipitated (Wang et al. 2011).

The crude polysaccharide extract was dissolved in water to a dilution of 40 mg/mL. Ethanol (0.1 volumes) was added to this solution (Wang et al. 2011). Precipitates were collected by filtration through a 400-mesh fabric and then freeze-dried. This fraction was named TMP-1. With the same process, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9 volumes of ethanol continued to be added to the supernatant. The resulting fractions were named TMP-2, -3, -4, -5, -6, -7, -8 and -9.

3.3 Purity and fractionation of polysaccharides

The molecular weight distribution and polysaccharide purity of these nine fractions were determined using a GPC-MALLS detector system. According to the results, choose the fraction with the highest polysaccharide content and relatively high polysaccharide purity for further study. TMP-5 was applied to a Sephacryl S-300 column (Majdoub et al. 2009). TMP-5 was further treated by Sephacryl S-300 chromatography (50 cm × 1.6 cm i.d.), with 46 cm packed height, ultrapure water eluting solvent and 0.5 mL/min flow velocity. The sample volume of TMP-5 is 1 mL with 50 mg/mL sample concentration. Furthermore, 0.2 mL of eluent was absorbed to monitor the polysaccharide content (Dubois et al. 1956). The remainder of the eluents were collected. As a result, two polysaccharide fractions were obtained, TMP-5I and TMP-5II. Given that TMP-5II had a greater available content; it was selected for subsequent structural studies.

3.4 Measurement of molecular weight of TMP-5II

Molecular weight was measured using GPC-MALLS. Waters 515 HPLC infusion system was adopted in the experiment with Shodex SB – G, Shodex SB – 804 and Shodex SB – 802.5 combination gel chromatography column and 18 angle laser light scattering instrument and
differential detector combination detector. The mobile phase is 0.1 mol/L sodium nitrate solution mixed with 0.02% solution of sodium azide, which was filtered using a 0.22 μm water filtration membrane. The sample volume is 0.2 mL with loading sample concentration of 0.1 mg/mL TMP-5II solution filtered by 0.45 μm water filter head. In the experiment, the flow rate was 0.5 mL/min, and the column temperature and detector temperature were both 45°C.

3.5 **Monosaccharide composition analysis**

The monosaccharide composition of the TMP-5II fraction was analysed by GC–MS. First, TMP-5II was hydrolysed with 2.0 M trifluoroacetic acid (Nelson & Cox 2004; Yu et al. 2009). Then, the hydrolysate was evaporated to dryness and restored. A small amount of acetic acid in methyl solution (0.1%, v/v) was added to remove excess KBH$_4$. Finally, the dried reduzate of acid hydrolysates was acetylated in acetic anhydride and pyridine (1:1, v/v) and converted into acetate derivatives dissolved in trichloromethane (Dong et al. 1995).

3.6 **IR spectroscopy**

IR analysis of TMP-5II was obtained on a Spectrum 400 infrared spectrophotometer (American PE company, Waltham, MA, USA) at a 4 cm$^{-1}$ resolution.

3.7 **NMR spectroscopy**

One-dimensional $^1$H NMR (500 MHz), $^{13}$C NMR (125 MHz) and 2D-NMR containing HMBC and HSQC spectra of the polysaccharide fraction (TMP-5II) were obtained with a Bruker AM-500 spectrometer (Bruker Company, Billerica, MA, USA).

4. **Conclusions**

The highly purified novel polysaccharide obtained from *T. matsutake*, TMP-5II, was determined to be a heteropolysaccharide. The present study also showed that TMP-5II consists of four monosaccharides, namely $\beta$-glucose, $\beta$-mannose, $\beta$-fucose and $\beta$-galactose, in the ratio of 10.52:1:2.08:7.52 according to GC–MS. Structural studies of TMP-5II demonstrated that the polysaccharide has a long backbone branching at O-6 and has a $\beta$ (1-6) linkage. In addition, an
α-linkage was indicated. From these results, we suspected that the polysaccharide has (1-4)-
beta-pyran glucose as a main chain, and a branched chain in the O-6 location with fucose (1-2)
mannose (1-3)-alpha-pyran galactose. The structure of the new isolated compound was achieved
(Figure 1).

The structure of the novel polysaccharide TMP-5II, a fraction of an extract from
T. matsutake with antitumour attributes, was elucidated. However, it is still hard to explain the
relationships between the chemical composition and structure characteristics and biological
functions for lacking mechanism study. Thus, further work will be focused on the structure and
biological activities of T. matsutake, which will offer insight into the mechanism of its
pharmacological activity.

Supplementary material
Supplementary material relating to this paper is available online at http://dx.doi.
org/10.1080/14786419.2015.1034711.

Acknowledgements
This work was supported by Projects of the National Science & Technology Pillar Program during the
Twelfth Five-year Plan Period (2011BAD23B00), and grants from China Cosmetic Collaborative and
Innovation Center (19005428069/007).

Disclosure statement
No potential conflict of interest was reported by the authors.

References
Chattopadhyay K, Adhikari U, Lerouge P, Ray B. 2007. Polysaccharides from Caulerpa racemosa: purification and
structural features. Carbohydr Polym. 68:407–415. doi:10.1016/j.carbpol.2006.12.010.
Dai Z, Zhang H, Zhang Y, Wang H. 2009. Chemical properties and immunostimulatory activity of a water-soluble
polysaccharide from the clam of Hyriopsis cumingii Lea. Carbohydr Polym. 77:365–369. doi:10.1016/j.carbpol.
2009.01.003.
Dong Q, Zhang ZY, Lin Y, Fang JN. 1995. Studies on two polysaccharides from Stephania tetrandra. Acta Biochim
Biophys Sin. 27:261–265.
Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and
related substances. Anal Chem. 28:350–356. doi:10.1021/ac60111a017.
Ebina T, Kubota T, Ogamo N, Matsunaga K. 2002. Antitumor effect of a peptide-glucan preparation extracted from a
mycelium of Tricholoma matsutake (S. Ito and Imai). Sing Biother. 16:255–259.
Ge Q, Zhang A, Sun P. 2009. Structural investigation of a novel water-soluble heteropolysaccharide from the fruiting
bodies of Phellinus baumii Pilát. Food Chem. 114:391–395. doi:10.1016/j.foodchem.2008.09.010.
Gong MQ, Su LJ, Chen Y, Wang FZ, Cao JX. 2002. A study on development of shiro and productive potentialities of
Tricholoma matsutake. For Res. 15:374–379.
Kim JY, Byeon SE, Lee YG, Lee JY, Park J, Hong EK, Cho JY. 2008. Immunostimulatory activities of polysaccharides
from liquid culture of pine-mushroom Tricholoma matsutake. J Microbiol Biotechnol. 18:95–103.
Liao LJ. 2010. Study on chemical composition and anti-tumor efficacy of Tricholoma matsutake [PhD dissertation].
Yanbian University.
Ma Z, Wang J, Zhang L. 2008. Structure and chain conformation of β-glucan isolated from Auricularia auricula-judae.
Biopolymers. 89:614–622. doi:10.1002/bip.20871.
Majdoub H, Mansour MB, Chaubet F, Roudesli MS, Maaroufi RM. 2009. Anticoagulant activity of a sulfated
polysaccharide from the green alga arthrosira platensis. Biochim Biophys Acta. 1790:1377–1381. doi:10.1016/
J.bbaggen.2009.07.013.
Mau JL, Lin HC, Song SF. 2002. Antioxidant properties of several specialty mushrooms. Food Res Int. 35:519–526.
doi:10.1016/S0969-9969(01)00150-8.
Nelson DL, Cox MM. 2004. Lehninger principles of biochemistry. New York (NY): Worth.
Snyder DS, Gibson D, Heiss C, Kay W, Azadi P. 2006. Structure of a capsular polysaccharide isolated from Salmonella enteritidis. Carbohydr Res. 341:2388–2397. doi:10.1016/j.carres.2006.06.010.

Wang LC, Wu H, Chang N, Zhang K. 2011. Anti-hyperglycemic effect of the polysaccharide fraction isolated from Mactra veneriformis. Front Chem Sci Eng. 5:238–244. doi:10.1007/s11705-010-0002-2.

Wang CW, Zhang K, Di LQ, Liu R, Wu H. 2011. Isolation and structural elucidation of novel homogenous polysaccharide from Mactra veneriformis. Carbohydr Polym. 86:982–987. doi:10.1016/j.carbpol.2011.05.052.

Wu M, Wu Y, Zhou J, Pan Y. 2009. Structural characterisation of a water-soluble polysaccharide with high branches from the leaves of Taxus chinensis var. mairei. Food Chem. 113:1020–1024. doi:10.1016/j.foodchem.2008.08.055.

Xiong W, Chen ZL, Zhong GH, Tian FY, Zheng L. 2009. Study on extraction of polyoses from Tricholoma matsutake in Tibet and their monosaccharide composition. Chem Bioeng. 26:58–61.

Yu R, Yin Y, Yang W, Ma W, Yang L, Chen X, Zhang Z, Ye B, Song L. 2009. Structural elucidation and biological activity of a novel polysaccharide by alkaline extraction from cultured Cordyceps militaris. Carbohydr Polym. 75:166–171. doi:10.1016/j.carbpol.2008.07.023.