Journal of Medicinal Plants Research Vol. 6(10), pp. 1935-1940, 16 March, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR11.1601
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Monosaccharide compositional analysis of purified polysaccharide from *Tricholoma matsutake* by capillary gas chromatography

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Accepted 3 February, 2012

Two polysaccharide (MTS-1 and MTS-2) were isolated from the water extract of *Tricholoma matsutake* and purified through ethanol precipitation, deproteination, DEAE-52 cellulose anion exchange and Sephadex G-100 gel-filtration chromatography. Their molecular weight was determined using high performance liquid chromatography and gel permeation chromatography (HPGPC) and their monosaccharide composition was analyzed by GC methods. It was shown that MTS-1 was composed of glucose, xylose, galactose in a molar ratio of 12.89:1.20:1 and MTS-2 consisted of glucose unit only. The molecular weights (Mw) of MTS-1 and MTS-2 were 5,939a and 232,317 Da, respectively. In the GC method for the analysis of monosaccharide composition of purified polysaccharide in *Tricholoma matsutake*, the peak area of each monosaccharide had a good linear relationship with its concentration (r^2>0.99). The intermediary precision values (expressed as the RSD) were less than 2%.The average recoveries of glucose, galactose and xylose ranged from 99.86 to 104.26% with RSD values of less than 5%. This developed method was accurate and had good reproducibility and could be used to determine the monosaccharide contents of *T. matsutake*.

Key words: *Tricholoma matsutake*, monosaccharide composition, gas chromatography.

INTRODUCTION

Mushrooms have been used widely since ancient times not only as foods or food-flavouring materials but also for medicinal and functional purposes. Research suggested they help in the treatment of certain types of cancer, boost the immune system and reduce the risk of coronary heart disease, because some of the edible mushroom species possess pharmacological properties (Kala, 2009).

Tricholoma matsutake (TM), which was an ectomycorrhizal parasite mushroom, belongs to the Basidiomycotina, Agaricales and Tricholomataceae families (Ohta, 1983). It was the most valuable one throughout the world and can be found in many regions of Africa, America, Europe and Asia (Redhead, 1997). In previous study, the wild edible mushroom contained rich sources of protein, fibre, carbohydrate and minerals showed that it was a nutritional fungus and had great value to health (Liu et al., 2010). Moreover, this mushroom exhibiting a characteristic and delicate flavour as well as several biological activities, such as cholesterol lowering, antioxidant, immunomodulating, and antitumor effects in humans (Hoshi et al., 2005; Mau et al., 2002). Its active components were considered to be polysaccharides (Hoshi et al., 2005; Ebina et al., 2002). Besides pharmacological properties and active components already available in the literature, little work has been carried out on the monosaccharide composition of polysaccharides from the mushroom. Therefore, in order to study the characterization of polysaccharides isolated from TM, the compositions of monosaccharide in the hydrolysates of TM polysaccharides were evaluated and validated.

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A variety of chromatographic systems may be used to separate and analyse the monosaccharides. Paper and thin-layer chromatography were the first chromatographic techniques used to separate individual sugars, but separations were limited with respect to the number of recognised analyses, presented poor resolution and were not always quantitative (Blanken et al., 1985). In recent years, high-performance liquid chromatography (HPLC) has been developed for analysis of monosaccharides (Dai et al., 2010; Giuseppe et al., 2001). Commonly, the methods for the HPLC analysis of carbohydrates use silica-based column packing materials with refractive index (RI) (Vonach et al., 1997) or short-wavelength ultraviolet (UV) (Nojiri et al., 2000; Fu et al.,1995) or fluorescence detection (Volpi, 2000). These methods show good performance with respect to selectivity and efficiency, but the main limitation was insufficient sensitivity and non-applicability to the RI detector.

The gas chromatography (GC) system was also valuable in the determination of monosaccharides by virtue of its excellent performance. Methods involving GC were rapid and sensitive (Chen et al., 2009; Guo et al., 2006). Excellent resolution and robustness were typically associated with GC applications owing to the large number of theoretical chromatographic plates and the inherent purity of the final derived sample dissolved in organic solvent. Typical detectors used for carbohydrate analysis include the flame ionisation detector (FID) and the mass selective detector (MSD) (John et al., 2005). Nevertheless, these methods require chemical transformation of the monosaccharides prior to the analysis in order to produce the necessary derivatives, which can be used to improve the chromatographic properties of the analysis and to improve its efficient trace determination. A large number of derivatisation reagents for monosaccharides have been reported in the literature including silylation reagents and acetylation reagents, such as hexamethyldisiazane (HMDS), trimethylchlorosilane (TMCS), N-methyl-N-(trimethylsilyl) trifluoroacetamid, trimethyl-silylimidazole (TMDS), heptafl uorobutyric anhydrides (HFBAA), pentafluoropropionic anhydrides (PFPA), hydroxylamine hydro-chloride/acetic anhydride/pyridine, trifluoroacetate (TFAA) etc (Joung et al., 2005; Merkle and Poppe, 1994). To determine the monosaccharide composition of a polysaccharide sample, it was usually hydrolysed completely, derivatised to promote volatility and finally analysed.

In the present study, we extracted, isolated and purified the polysaccharide from TM and the compositions of monosaccharides in the hydrolysates of polysaccharide were evaluated and validated using a GC method. In a chromatographic system employing a FID detector, monosaccharides were derivatised into trimethylsilylation and separated on a HP-50 capillary column under optimised chromatographic conditions. In order to develop an accurate method for quantification, a data analysis method with an internal standard was used. With the chromatographic conditions employed, excellent resolution of the common monosaccharides and the internal standard was obtained in 16 min. The present study demonstrated that this method could be employed in the reproducible quantification of monosaccharides and has the advantage of speed, sensitivity, and reproducibility.

MATERIALS AND METHODS

Chemicals and apparatus

TM samples were collected from Yunnan province in southwest China. The collected samples were cleaned, cut and freeze-dried. Dried samples were crushed by using a mortar with pestle and stored in pre-cleaned polyethylene bottles until the analysis started.

Standard reagents including D-glucose (Glu), D-galactose (Gala), D-arabinose (Arab) , D-xylose (Xyl), D-rhamnose (Rha), D-mannose (Man), glucuronic acid (GluA) and Dextran standards (4,000, 20,000, 120,000, 190,000, 270,000 and 400,000 Da) were purchased from Sigma (St. Louis, MO, USA). All other chemicals and solvents used were of analytical grade and purchased from Shanghai Chemicals and Reagents Co. (Shanghai, China). Sephadex G-100 and DEAE-cellulose were from Sinopharm Chemical Reagent. Co. Ltd. Ultrapure water was obtained from a Millipore (Billerica, MA, USA) water purification system equipped with a multi-wavelength UV lamp and an organic acid polishing cartridge. The polysaccharide content of the different eluting fractions was monitored using the phenol-sulfuric acid method, with an UV-Vis spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., P.R. China).

GC analyses were performed with a 6890 N Net work GC System (Agilent Technologies, USA) gas chromatograph with a HP-50 capillary column (30 m, 0.32 mm i.d.; 0.25 μm film thickness) and a flame ionisation detector.

Isolation and purification

Conventional procedures were followed for the isolation of polysaccharides (Chen et al., 2009). The powders of TM were soaked with 95% ethanol at room temperature to remove the pigments and small lipophilic molecules. The residue was then extracted with 10 vol. of double-distilled water at 90°C for 3 h thrice and filtered. The combined aqueous extracts were concentrated in a rotary evaporator under reduced pressure at 50°C and filtered. Then the filtrate was precipitated by the addition of 95% ethanol (4 times the volume of aqueous extract) at 4°C for overnight, followed by centrifugation at 4500 rpm (15 min). The precipitate was dissolved in water and deproteinated with Sevage reagents ($CHCl_3$–n-BuOH with v/v = 4:1) until the protein absorption peak was not detected by UV. Finally the supernatant was lyophilized to give the crude polysaccharides.

The crude polysaccharides were purified by DEAE-52 cellulose and Sephadex G-100, and the main polysaccharide fractions (MTS-1,MTS-2) were collected, lyophilized and used for further study.

Determination of molecular weight

Dextran standards, MTS-1 and MTS-2 were dissolved in distilled water at a concentration of 2.0 mg/ml and analyzed on an Agilent 1100 Series HPLC system equipped with a RID and a TSK G5000PWXL gel column (7.8 × 300 mm) (TOSOH Corporation, Japan) to determine the retention time of standards and samples

(Du et al., 2008). The column and detector compartment were maintained at 30 and 35°C, respectively. Distilled water was used as mobile phase at a flow rate of 1.0 ml/min and injection volume was 10 µl. The molecular weight of MTS-1 and MTS-2 were calculated by constructing a calibration curve, in which the logarithm of the molecular weight of the Dextran standards ranging from 4,000 to 400,000 Da was plotted as a function of the retention time using Agilent ChemStation GPC Data Analysis Software (Rev. A.02.01).

Analysis of monosaccharide compositions

MTS-1 and MTS-2 (10 mg) were hydrolysed by 10 ml of 2 M trifluoroacetic acid (TFA) at 100°C for 4 h (Yang et al., 2009), respectively. After removing TFA with methanol, derivatisation of the released monosaccharides was then carried out using the trimethylsilylation reagent according to the method of Guentas et al. (2001). The trimethylsilylated derivatives were loaded onto a HP-50 capillary column and determined by a flame ionisation detector. The following program was adopted for gas chromatography analysis: Injection temperature: 295°C; detector temperature: 300°C; column temperature programmed from 130 to 290°C at 10°C/min. Nitrogen was used as the carrier gas and maintained at 1.0 ml/min.

Inositol was used as the internal standard. The correction factor for each monosaccharide was calculated using Equation (1):

$$ƒi/s = (Wi/Ws)/(Ai/As) \quad (1)$$

where As and Ai are the peak areas of the internal inositol standard and the standard monosaccharide in the reference solution, respectively; Ws and Wi are the content of the internal inositol standard and the standard monosaccharide in the reference solution, respectively.

The content of every monosaccharide in the polysaccharide hydrolysis solution (W) was calculated with Equation (2):

$$W = ƒi/s(Ai/As)Ws \quad (2)$$

where As and Ai are the peak areas of the internal standard inositol and the monosaccharide in the polysaccharide reducing solution, respectively, and Ws is the content of the internal standard inositol in the polysaccharide hydrolysis solution.

Method validation

The method was validated for parameters such as linearity, precision, accuracy and stability following the International Conference on Harmonization (ICH) guidelines (The European Agency for the Evaluation of Medical Products, 1996). The calibration curves were constructed by plotting the peak area versus the concentration of each analyte. The square of the correlation coefficients (r^2) were determined by linear regression. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively. The magnitude of analytical background response was measured by injection of blank samples and the S/N ratio for each compound was calculated by injecting a series of diluted solutions. Intra- and inter-day variations were chosen to determine the precision of the developed assay and expressed by relative standard deviation (RSD). The recovery was used to evaluate the accuracy of the method. Various amounts of each analyte were added to a variety of samples, and the resulting spiked samples were subjected to the entire analytical sequence. Each analyte was spiked at three different concentrations (high, medium and low levels of the calibration range). Three replicates were performed for the test and the recovery was calculated according to the following formula: recovery(%) = (total amount after spiking original amount in sample)/spiked amount × 100%.To confirm the stability, the same real sample was analyzed within 24 h and at room temperature. The RSD value was calculated as a measurement of stability and method repeatability.

RESULTS AND DISCUSSION

Molecular weight

A HPLC-GPC method was used to determine the molecular weight of MTS-1 and MTS-2. The molecular weight (Mw) of MTS-1 and MTS-2, calculated using GPC software, were 5,939 and 232,372Da, respectively. The Dextran calibration curve was LogMw = 11.0335-0.2342T(T: retention time of Dextran).

GC chromatography

Three typical chromatograms are shown in Figure 1 and the elution times and peak areas are tabulated in Table 1. Under the present chromatographic condition, all derivatised monosaccharide were obvious baseline separation and the peaks for all monosaccharides were sharp and symmetrical in chromatogram. The retention times of Ara (arabinos), Rham (rhamnose), Xyl (xylose), Man (mannose), Gal. (galactose), Glu (glucose), Inositol were 7.580, 7.696, 8.608, 9.514, 10.059, 10.407 and 10.545 min, respectively.

According to GC analysis, polysaccharide MTS-1 from TM contain glucose, xylose and galactose in a molar ratio: Glu:Xyl:Gal = 12.89:1.20:1. The composition of monosaccharide in MTS-2 was only glucose units. The use of a 30 m × 0.32 mm capillary column resulted in good resolution with a run time of 16 min; hence the described method has the advantages of simplicity and a short run time, without any loss of analyte, and with a small sample volume. The overall time of analyses was more acceptable for routine analyses than that of Chen et al. (2009). The use of internal standard helped in monitoring the recovery of monosaccharides from TM polysaccharide.

Linearity, range and limits of detection

The linearity of the plot Ai/As (X) for each monosaccharide vs peak areas ratios Wi/Ws (Y) was investigated; the results were expressed as the values of square of the correlation coefficient (R^2) in Table 2. All six GC calibration curves of the monosaccharides, Arab, Rha, Xyl, Man, Gala and Glu exhibited good linearity with excellent correlation coefficients. The square of the correlation coefficients were greater than 0.99, which showed a good linearity within the test concentration range. The common linear range for all six monosaccharides was between 0.071 and 2.4 mg/ml.

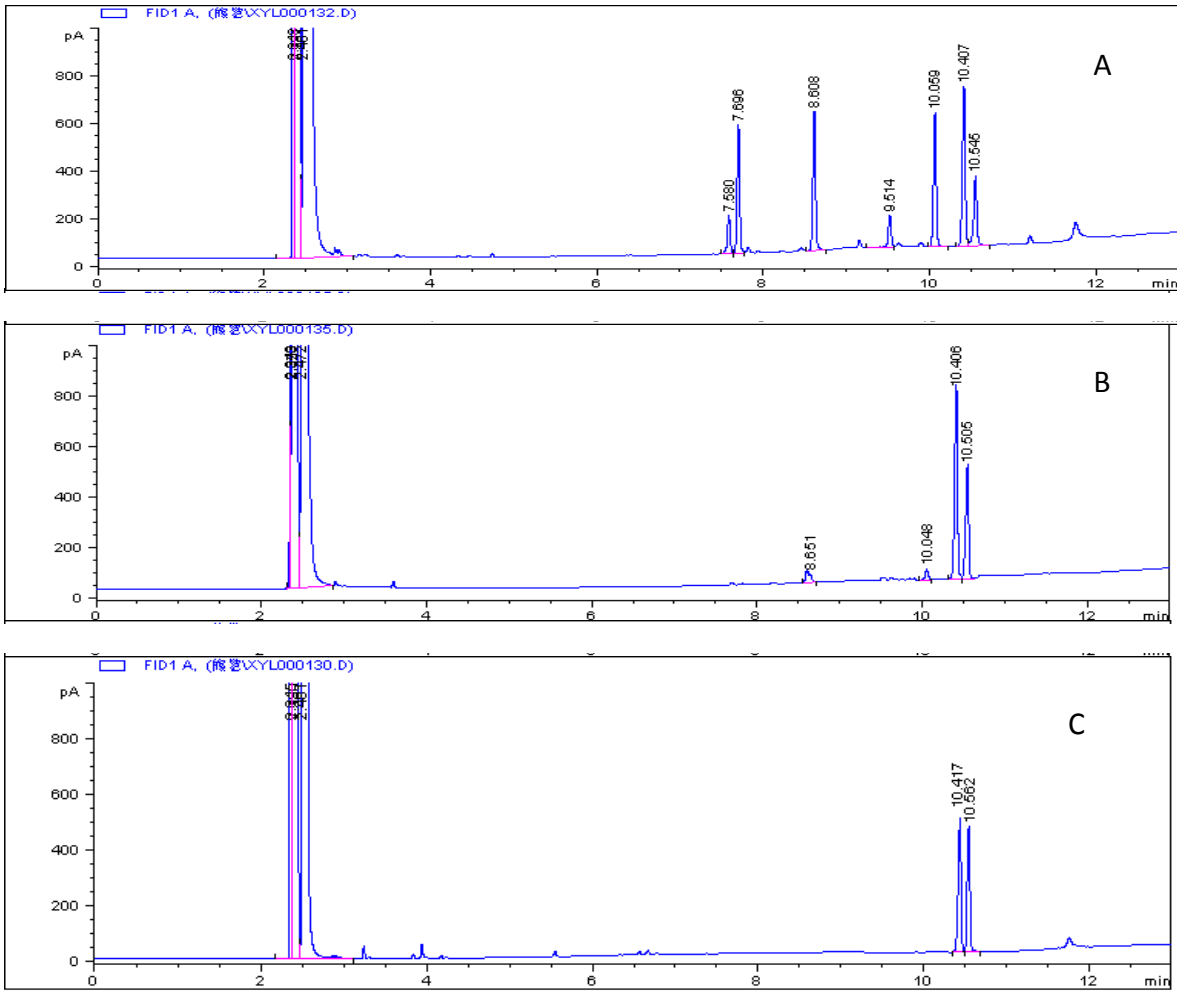

Figure 1. GC chromatograms of standards (A), along with sample MTS-1(B) and MTS-2(C).

Table 1. GC data of monosaccharides in standard mixture as their derivatisation.

Monosaccharide	Peak	Retention time(min)	Peak area
D-Arabinose (Ara)	1	7.580	382.517
D-Rhamnose (Rham)	2	7.696	1486.687
D-Xylose (Xyl)	3	8.608	1552.813
D-Mannose (Man)	4	9.514	362.074
D-Galactose (Gala)	5	10.059	1501.937
D-Glucose (Glu)	6	10.407	1677.948
Inositol	7	10.545	692.967

LOD and LOQ values are reported in Table 2 for each monosaccharide. Accordingly, the detection limits were 0.0145, 0.0136 and 0.0151 mg/ml for Xyl, Gala and Glu, respectively. The results demonstrated that the internal standard method was suitable for the analysis of the composition of the monosaccharides in the polysaccharide.

Precision and stability

The instrument precision was examined by the performance of the intra-day and inter-day assays by six replicated injections of the mixture standard solutions at medium concentration. The intra-day precision was performed with the interval of 4 h in the same day, and

Table 2. Linear relation between peak area and concentration.

Monosaccharide	Regression equation	R^2	LOD (mg/ml)	LOQ (mg/ml)
D-Arabinose (Ara)	Y = 0.4127X - 0.2031	0.9987	0.0251	0.0751
D-Rhamnose (Rham)	Y = 0.5373X - 0.0025	0.9982	0.0125	0.0465
D-Xylose (Xyl)	Y = 0.4384X + 0.0126	0.9977	0.0145	0.0566
D-Mannose (Man)	Y = 0.4210X - 0.1680	0.9989	0.0283	0.0725
D-Galactose (Gala)	Y = 0.4023X + 0.1124	0.9981	0.0136	0.0482
D-Glucose (Glu)	Y = 0.4136X - 0.1026	0.9993	0.0151	0.0548

Table 3. Recoveries of the three monosaccharides in sample (n = 3).

Monosaccharide	Added (mg/ml)	Detected (mg/ml)	Recovery (%)[a]	RSD (%)
D-Xylose (Xyl)	0.5	0.5063	101.26	2.64
	1.0	1.0098	100.98	4.23
	2.0	2.0670	102.35	2.34
D-Galactose (Gala)	0.5	0.4993	99.86	1.85
	1.0	1.0136	101.36	3.24
	2.0	2.0112	100.56	1.42
D-Glucose (Glu)	0.5	0.5213	104.26	4.85
	1.0	1.0265	102.65	1.68
	2.0	2.0206	101.03	2.11

[a], Data are means of three experiments.

the inter-day precision was performed over 3 days. The precision results showed that the RSD values of peak area ranged from 0.86 to 1.87% both for the intra-day and inter-day precision. The same real sample was analyzed for the stability within 24 h and at the room temperature. The results demonstrated that the analytes were stable in the conditions, and RSD values of peak area of Xyl, Gala and Glu were 1.83, 1.45 and 1.96%, respectively.

Recovery test

The average percentage recovery of Xyl, Gala and Glu were determined and these ranged from 99.86 to 104.26% with RSD values of less than 5% (Table 3). As a whole, the results clearly indicated that the level of monosaccharide present did not affect the percentage recovery: the level was within the linear range and the interference of the matrix with respect to the percentage recovery was also very low. Considering the results of the recovery test, the method was deemed to be accurate.

Conclusion

The analysis conditions of monosaccharide composition of the polysaccharides using GC method based on trimethylsilylation reagent pre-column derivatization was detailedly examined and optimized. The modified GC method of pre-column derivatization was suitable to some minute quantities of polysaccharide samples with high resolution separation and simultaneous determination of many kinds of component sugars. Monosaccharide compositions of the polysaccharide fractions from TM were determined by the modified method. The results showed that MTS-1 was composed of glucose, xylose, galactose in a molar ratio of 12.89:1.20:1 and MTS-2 consisted of glucose unit only. The molecular weights (Mw) of MTS-1 and MTS-2 were 5,939 and 232,317 Da, respectively.

Based on the neutral sugar analysis, many international researches suggested that polysaccharides of medicinal plants composed of glucan were known to stimulate the immune system and have perfect anticancer effects. Up to now, many of those herbal plants have been universally made into medicines, such as *Lentinus edodes* (shiitake) polysaccharide and Tuckahoe polysaccharide (Chen, 1997). Moreover, polysaccharides of plants consisting of Gala, Glu, GalA and Rha are also said to have powerful anti-bacteria, anti-infl ammation and anti-virus properties, such as *Althaea officinalis* var. *rhobusta* polysaccharide (Peter et al., 1987).

However, there have been few reports on the heter-opolysaccharide consisting of Glu, Gala and Xyl.

Therefore it is necessary to study the relationship between the structure of the polysaccharide and its pharmacological activities. Further studies on the pharmacological activities of polysaccharides and the potencies of TM extracts are currently in progress in our laboratory.

ACKNOWLEDGEMENTS

The financial support for this study by National Natural Science Fund of China(No: 31100253), Natural Science Fund of Hubei Provincial (No:2010CDB06907) and Wuhan University Project Fund (No:302274658) is gratefully acknowledged.

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