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Characterization of water and alkali-soluble polysaccharides from *Pleurotus tuber-regium* sclerotia

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ABSTRACT

Pleurotus tuber-regium sclerotia, an edible and medicinal mushroom, is a rich source of polysaccharides used as a functional food to promote health and longevity. The crude polysaccharides were isolated from the *P. tuber-regium* by hot aqueous and alkali extraction and then further purified by DEAE-52 cellulose chromatography and Sephadex G-100 chromatography. Two polysaccharides, water and alkaline extraction of polysaccharides (W-PTR and A-PTR), were obtained and their extraction process were optimized through orthogonal array design. Structure characteristics (physicochemical property analysis, molecular weight, monosaccharide composition, sulfate and uronic acid contents, triple helical structures, ultraviolet spectrum and infrared spectroscopy) of the two polysaccharides were investigated. Results showed that the main difference between the two polysaccharides is reflected in color, solubility, molecular weight and monosaccharide composition. Conformational analysis showed that both W-PTP and A-PTP had triple-helix conformation. The 3D structure of the two polysaccharides and their structure–function relationship will be challenge in the future.

1. Introduction

Nowadays, mushrooms have attracted much attention as a functional food and as a source for the development of drugs and nutraceuticals. In many Asian countries, mushrooms such as lingzhi (*Ganoderma lucidum*), shiitake (*Lentinus edodes*), and yiner (*Tremella fuciformis*) that have been collected, cultivated and used for hundreds of years are being evaluated as edible and medicinal resources (Hobbs, 2000). Polysaccharides are the best known and most potent mushroom-derived substances with a number of medicinal properties.

Pleurotus tuber-regium, an edible and medicinal mushroom native to tropical and subtropical regions, which belongs to the family Pleurotaceae in the phylum Basidiomycota, was first discovered in Africa and used as a functional food to promote health and longevity (Zoberi, 1973). *P. tuber-regium* has different morphological forms including fruit body, sclerotium, and mycelium which contain various bioactive substances such as glycoproteins and polysaccharides as well as phytochemicals that have pharmacological and nutritional values (Wong & Cheung, 2008). The nonstarch polysaccharides extracted from the sclerotium of *P. tuber-regium* have been demonstrated to have both immunomodulatory and direct cytocidal antitumor activities (Wong, Wong, Chiu, & Cheung, 2007; Zhang, Cheung, & Zhang, 2001; Zhang, Zhang, & Cheung, 2004).

Several water-soluble and water-insoluble glucan were isolated from both the hot aqueous and alkali extracts of *P. tuber-regium* sclerotium. There are studies on the extraction of active constituents from *P. tuber-regium*, but no researches relating to the extraction optimization of alkaline extraction polysaccharides (A-PTR) and the comparison between A-PTR and water extraction polysaccharides (W-PTR) from *P. tuber-regium* have been reported. In this study, two different crude polysaccharides isolated from the hot aqueous and alkali extracts were investigated and their chemical characteristics were characterized. W-CPTP and A-CPTP polysaccharides were further isolated with DEAE-52 cellulose chromatography and Sephadex G-100 chromatography. Meantime, in order to understand the structure difference between W-PTP and A-PTP, their molecular weights, monosaccharide compositions, sulfate and uronic acid contents, triple helical structure were analyzed and compared as well.

2. Materials and methods

2.1. Materials and reagents

The *P. tuber-regium* sclerotia were purchased from Shantou Light Industry Co. Ltd. Thai bacteria (Guangdong, China).

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Table 1
Orthogonal experiment design for the extraction of polysaccharides with hot water and alkali solution.

Level	Factor					
	Hot water extraction			Alkali solution extraction		
	Extraction temperature	Extraction time (h)	Solid/liquid ratio	Solid/liquid ratio	NaOH concentration (mol/l)	Extraction time (h)
1	80	2	1:30	1:4	0.3	1.0
2	90	3	1:40	1:6	0.5	1.5
3	100	4	1:50	1:8	0.7	2.0

3,5-Dinitrosalicylic acid (DNS), bovine albumin (BSA), Coomassie brilliant blue, carbazole, glucuronic acid, hydroxylamine hydrochloride, pyridine, Congo red were purchased from Sigma Chemical Co. (America). Sephadex G-100 column was from Pharmacia Co. (America). The standard monosaccharides (sorbose, arabinose, xylose, glucose, galactose and glucuronic acid) purchased from Shanghai Bo'ao Biological Technology Co. Ltd. (China). Dextrans of different molecular weights were from Sigma Chemical Co. (America). All other chemicals and solvents were of analytical grade from China.

2.2. Extraction parameters for water-soluble/alkali-soluble crude polysaccharide

Dried *P. tuber-regium* powder was extracted based on previously reported methods with some modifications (Ahrazem, Leal, & Prieto, 2001; Gutierrez, Prieto, & Martinez, 1996; Nie & Ning, 2004). Hot water method: the pre-prepared powder (10 g) was treated with the temperature of the water bath ranging from 80 to 100 °C, and different extract time (2 h, 3 h, and 4 h) with a ratio of solid/liquid (g/ml) (ranging from 1:30 to 1:50).

Alkali solution method involved different NaOH concentrations (0.3, 0.5, and 0.7 mol/l), and different extract time (1 h, 1.5 h, and 2 h) with a ratio of solid/liquid (g/ml) (ranging from 1:4 to 1:8). After filtration through 0.45 mm filter paper, the filtrates were combined and concentrated using a rotary evaporator at 45 °C, and then freeze-dried in vacuum. The dry powder obtained was dissolved by distilled water to a constant volume till analysis. The samples were measured at 490 nm and 540 nm by UV–vis spectrophotometer to represent the content of total sugar and reducing sugar, respectively. The effects of different parameters on the polysaccharide yield could be obtained.

An orthogonal test is a method that analyzes multiple factors using orthogonal tables. Comprehensive understanding of the test can be obtained through implementation, and the primary and secondary factors can be determined using statistical analysis. The orthogonal experimental design can ensure not only that the experimental parameters are optimized, but also that the workload is greatly reduced.

An orthogonal $L(3)^3$ array design was used to investigate the optimal extraction condition of polysaccharides from *P. tuber-regium* (Table 1).

2.3. Preparation of crude polysaccharides

Fresh *P. tuber-regium* sclerotia was dried at 60 °C, powdered and sieved through a No. 300 mesh. The *P. tuber-regium* sclerotia powders were extracted with hot water/alkali solution, and the extraction was carried out under the optimized conditions, respectively. Crude polysaccharide extract was deproteinized with Sevag reagent (chloroform:butanol, 4:1), and then precipitated with 4-fold volume ethanol (75%). After centrifugation at 5000 rpm for 15 min, the precipitate was washed successively with ethanol (75%) and acetone, dialyzed against deionized water, and lyophilized as crude polysaccharides, namely W-CPTP and A-CPTP.

2.4. Deproteinization

Crude polysaccharide was mixed with Sevag reagent (chloroform:butanol, 4:1, v/v) at a ratio of 3:1 (v/v). The mixture was allowed to react at room temperature for 40 min. After centrifugation for 10 min at a speed of 10,000 rpm, the supernatant was used for the analysis of deproteinization.

2.5. Isolation and purification of the polysaccharides

W-CPTP/A-CPTP was dissolved in distilled water, and then filtered through 0.45 μm membrane. The W-CPTP/A-CPTP solution was subjected to a DEAE-52 cellulose column (2.6 cm × 50 cm) with a stepwise elution of NaCl solution (0, 0.1, 0.3, and 0.5 mol/l). The eluates were combined according to the total carbohydrate content quantified by phenol–sulfuric acid method to afford W-CPTP-1/A-CPTP-1. After dialyzing and concentrating, W-CPTP-1/A-CPTP-1 was further purified with a Sephadex G-100 column (2.6 cm × 60 cm). Distilled water served as the eluants at a flow rate of 0.4 ml/min. Finally, W-PTP and A-PTP were obtained.

2.6. Determination of total sugar, reducing sugar, protein, uronic acid and sulfate contents

The total sugar content was determined by the phenol–H_2SO_4 method, with glucose as the standard at 490 nm (Peng, Zhang, Zeng, & Xu, 2003). The reducing sugar content was determined by the dinitrosalicylic acid (DNS) method, with glucose as the standard at 540 nm (Kim, Park, & Nam, 2003). Protein was measured by Bradford's method (Bradford, 1976) using bovine serum albumin (BSA) as the standard at 595 nm. The content of uronic acid in polysaccharide was determined by measuring the absorbance at 530 nm using sulfuric acid–carbazole method with glucuronic acid as the standard (Karamanos, Hjerpe, Tsegenidis, Engfeldt, & Antonopoulos, 1988). The content of sulfate groups in polysaccharide was measured by the barium chloride–gelatin method using potassium sulfate as the standard at 360 nm (Cong, Wang, Li, & Wu, 2003). The following equation was used to quantify the polysaccharide yield:

$$\text{polysaccharide yield}\,(\%) = 0.9 \times (S - R) \times 100 \quad (1)$$

where S is the total sugar content (%), R is the reducing sugar content (%) and 0.9 is conversion coefficient of glucose convert to polysaccharide.

2.7. Physicochemical property analysis

Physicochemical properties were determined using the following method: color observation, solubility test, ninhydrin reaction, iodination reaction, Folin–Wu reaction, Benedict reaction, sulfuric acid–carbazole reaction, $FeCl_3$ reaction and Coomassie brilliant blue reaction.

2.8. Determination of molecular weight

The homogeneity and molecular weight of W-PTP/A-PTP was determined by high performance gel permeation chromatography (HPGPC), which was performed on a Waters HPLC system (717 Plus sample injector, 152 pump, Waters) equipped with two gel columns (TSK PWXL G-5000 and G-3000) coupled with a refractive index detector (Waters 2410). A sample solution (20 μl of 1.0 mg/ml polysaccharide) was injected in each run, eluted with 0.02 mol/l KH_2PO_4 (PH 6.0) as the mobile phase at a flow rate of 0.6 ml/min.

The molecular weight of the purified polysaccharide was determined by a gel chromatography. T-series standard dextran of known molecular weights (3300, 18,300, 100,000, 164,000, 236,000, 333,000 and 1223,000) was passed through the column with same analysis condition as W-PTP and A-PTP. Then the elution volumes were plotted against the logarithm of their respective molecular weights. The calibration curve of $\log M_w$ (molecular weight) of standard dextrans on GPC against their elution volume (V) was obtained ($\log M_w = 1.03e{+}002 - 1.86e{+}001V + 1.18e{+}000V^2 - 2.51e{-}002V^3$, $R^2 = 0.9981$). The elution volume of the purified polysaccharide was plotted in the same calibration curve, and the molecular weight was determined (Yoo, Yoon, Cha, & Lee, 2004).

2.9. Conformational analysis

The conformational structure of the polysaccharides in solution was determined by observing the bathochromic shift of the visible maximum absorption of Congo red–polysaccharide complexes (Rout, Mondal, Chakraborty, & Islam, 2008). The transition from a triple-helical arrangement to the single-stranded conformation was proved by measuring the maximum absorption wavelength (λ_{max}) of Congo red–polysaccharides solutions at different concentrations of NaOH (de Nooy, Rori, Masci, & Dentini, 2000; Leung, Fung, & Choy, 1997). Briefly, 6 mg of polysaccharides was dissolved in 2.0 ml of distilled water and reacted with 2.0 ml 100 μmol/l of Congo red in a gradient of sodium hydroxide solutions (0.0, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5 and 1.0 mol/l). The absorbance was measured in the range of 400–600 nm, and λ_{max} at different concentrations of sodium hydroxide was plotted. Distilled water without adding polysaccharide was served as the control.

2.10. Monosaccharide composition analysis

Monosaccharide composition of W-PTP/A-PTP was analyzed by gas chromatography (GC) as described by Sheng et al. (2007) with some slight modifications. Briefly, 10 g of W-PTP/A-PTP was hydrolyzed with 4 ml 2 mol/l of trifluoroacetic acid (TFA) at 110 °C for 2 h. After removing the residual TFA with methanol under reduced pressure, the sample was dissolved in 1 ml of pyridine and reacted with 10 mg of hydroxylamine hydrochloride for 30 min at 90 °C. Afterwards, 1 ml of acetic anhydride was added and incubated for another 30 min at 90 °C. Six standard monosaccharides, sorbose ($M_w = 180$), arabinose ($M_w = 150$), xylose ($M_w = 150$), glucose ($M_w = 180$), galactose ($M_w = 180$) and glucuronic acid ($M_w = 194$), were converted to their acetylated derivatives according to the above-mentioned method.

Sample derivatives were analyzed by GC using an Agilent HP4890D instrument equipped with an HP-5 capillary column (30 m × 0.32 mm × 0.25 μm) and a flame ionization detector (FID). The applied temperature program was as follows: oven temperature was initially set at 170 °C, increasing to 250 °C at a rate of 5 °C/min. The heater temperatures of both injector and detector were kept at 250 °C. Nitrogen was used as the carrier gas. The relative molar proportions of sugars in polysaccharide were calculated by the area normalization method according to the chromatogram (Deng, Liu, Li, & Yu, 2000).

2.11. Infrared spectroscopy

The polysaccharide (2 mg) was mixed with spectroscopic grade potassium bromide (KBr) powder, ground and pressed into a 1 mm pellets for Fourier transform infrared (FI-IR) measurement at room temperature. A FI-IR spectrum of the W-PTP/A-PTP was determined using the Vector-33 spectrometer in the frequency range of 4000–500 cm^{-1} (Ding, Zhang, & Zhang, 2004).

2.12. Statistical analysis

All experiments were carried out in triplicate and data were expressed as mean ± standard deviation. Statistical analysis was performed with one-way analysis of variance (ANOVA). Values of $P < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Optimization of the extraction parameters of polysaccharides

Owing to the many factors that influence extraction of polysaccharides, optimization of the extraction protocol is required. The combination effects on the extraction of polysaccharides were evaluated through an orthogonal experiment ($L_9(3)^3$) in order to detect the most suitable extraction conditions (Table 1). In view of the orthogonal analysis, we adopt statistical software to calculate the values of K (range analysis) and R (variance analysis) (Li & Jiang, 2010), and the analysis results are presented in Table 2.

For the hot water extraction, it can be seen from Table 2 that the extraction temperature plays the most important role in the extraction of polysaccharides, and the order of importance that influenced the extraction rate was found to be: extraction temperature > solid/liquid ratio > extraction time according to their significant role in the degree (R values). The optimum combination of variables was $A_3B_1C_1$; that is, extraction temperature 100 °C, solid/liquid ratio 1:30, and extraction time 2 h.

For the alkali solution extraction, It can be seen that the order of importance that influenced the extraction rate was found to be: extraction time > solid/liquid ratio > NaOH concentration according to their significant role in the degree (R values). The optimum combination of variables was $A_2B_2C_3$; that is, solid/liquid ratio 1:6, NaOH concentration 0.5 mol/l, and extraction time 2 h.

3.2. Isolation and purification of crude polysaccharides

In the present study, W-CPTP/A-CPTP was isolated from *P. tuberregium* and the yield was about 1.42%/0.448%, which is lower than that of precious literature (Wong et al., 2007). Furthermore, the W-CPTP/A-CPTP solution was firstly separated through an anion-exchange chromatography of DEAE-52, affording an independent elution peak as detected by the phenol–sulfuric acid assay. The fraction were collected, concentrated and purified by gel filtration chromatography of Sephadex G-100. As a result, both of W-CPTP and A-CPTP generated two peaks. The first larger peak has high polysaccharide content and low protein content, while on the contrary, the second small peak has low polysaccharide content and high protein content. So the second peak was removed and the first large peak was obtained as W-PTP and A-PTP. After concentration, the collected fractions were dialyzed and lyophilized for subsequent research.

Table 2
Orthogonal experiment results of hot water and alkali solution extraction and difference analysis between factor levels of extraction yield.

Trial	Hot water extraction				Alkali solution extraction			
	A Extraction temperature	*B* Extraction time (h)	*C* Solid/liquid ratio	Extraction yield (%)	*A* Solid/liquid ratio	*B* NaOH (mol/l)	*C* Extraction time (h)	Extraction yield (%)
1	80 (A_1)	2 (B_1)	1:40 (C_2)	1.04 ± 0.04	1:4 (A_1)	0.3 (B_1)	1 (C_1)	0.166 ± 0.014
2	80 (A_1)	3 ($B2$)	1:50 (C_3)	1.20 ± 0.12	1:4 (A_1)	0.5 (B_2)	1.5 (C_2)	0.426 ± 0.134
3	80 (A_1)	4 (B_3)	1:30 (C_1)	0.93 ± 0.09	1:4 (A_1)	0.7 (B_3)	2 (C_3)	0.438 ± 0.138
4	90 (A_2)	2 (B_1)	1:50 (C_3)	1.31 ± 0.10	1:6 (A_2)	0.3 (B_1)	1.5 (C_2)	0.424 ± 0.082
5	90 (A_2)	3 (B_2)	1:30 (C_1)	1.06 ± 0.16	1:6 (A_2)	0.5 (B_2)	2 (C_3)	0.448 ± 0.064
6	90 (A_2)	4 (B_3)	1:40 (C_2)	1.15 ± 0.15	1:6 (A_2)	0.7 (B_3)	1 (C_1)	0.278 ± 0.156
7	100 (A_3)	2 (B_1)	1:30 (C_1)	1.42 ± 0.04	1:8 (A_3)	0.3 (B_1)	2 (C_3)	0.438 ± 0.080
8	100 (A_3)	3 (B_2)	1:40 (C_2)	1.58 ± 0.42	1:8 (A_3)	0.5 (B_2)	1 (C_1)	0.234 ± 0.016
9	100 (A_3)	4 (B_3)	1:50 (C_3)	1.77 ± 0.14	1:8 (A_3)	0.7 (B_3)	1.5 (C_2)	0.274 ± 0.044
K_1	3.17	3.77	3.41		1.030	1.028	0.678	
K_2	3.52	3.85	3.78		1.150	1.108	1.124	
K_3	4.78	3.85	4.27		0.946	0.990	1.134	
k_1	1.06	1.26	1.14		0.344	0.342	0.226	
k_2	1.17	1.28	1.26		0.384	0.370	0.374	
k_3	1.59	1.28	1.42		0.316	0.33	0.442	
R	0.54	0.02	0.29		0.040	0.028	0.068	

3.3. Deproteinization

Many methods have been carried out to remove the protein impurities, including Sevag method, trichloroacetic acid method and enzymolysis (You, Xie, Liu, & Gu, 2010). Sevag method is complicated and time-consuming. Fig. 1 shows the deproteinization of Sevag method on the polysaccharides. We found that the yield of polysaccharide was gradually decreased with the increasing deproteinization time within a certain range, while the rate of deprotein was increased. The more deproteinization times, the much loss of polysaccharide. In order to improve efficiency and obtain the maximum polysaccharides (W-PTP and A-PTP), the deproteinization time were both set at 3 was appropriate.

3.4. Physicochemical property

Table 3 lists the physicochemical properties of the two polysaccharides. The colors of the two polysaccharides extracted by different methods had a few differences. W-PTP was white, whereas A-PTP was gray. W-PTP was water soluble especially in hot-water, it has strong moisture absorption and can be dissolved in dimethyl sulfoxide (DMSO) but has little solubility in other organic solvent such as high concentration of methanol, ethanol, acetone, ethyl acetate, and butyl alcohol. A-PTP was alkali soluble and it can be dissolved in hot water but much weaker than W-PTP, it cannot be dissolved in organic solvent such as high concentration of methanol, ethanol, acetone, ethyl acetate, and butyl alcohol. The results of the Coomassie brilliant blue, ninhydrin, iodination, Folin–Wu, Benedict, $FeCl_3$, and sulfuric acid–carbazole tests were similar for the two polysaccharides. These similarities indicated that the two extractions were polysaccharides and contained uronic acid, but did not contain protein, starch, small peptide, free amino acid, free monosaccharide, free reducing sugar or polyphenol (Wu, Zhu, Zhang, Yang, & Zhou, 2012).

The total sugar content of W-PTP (0.788 mg/ml) was almost double of that of A-PTP (0.478 mg/ml) (Table 3). Consistently, A-PTP had much higher protein content (0.035 mg/ml) than that of W-PTP (0.008 mg/ml).

3.5. Molecular weight

The HPGPC profile showed a single and symmetrically sharp peak, indicating that W-PTP/A-PTP was a homogeneous polysaccharide (Jahanbin, Gohari, Moini, Emam-Djomeh, & Masi, 2011). According to the calibration curve with standard dextrans, the number-average molecular weight (M_n) and the weight-average molecular weight (M_w) of polysaccharide were obtained. GPC chromatogram of W-PTP and A-PTP is showed in Fig. 2. It can be seen that

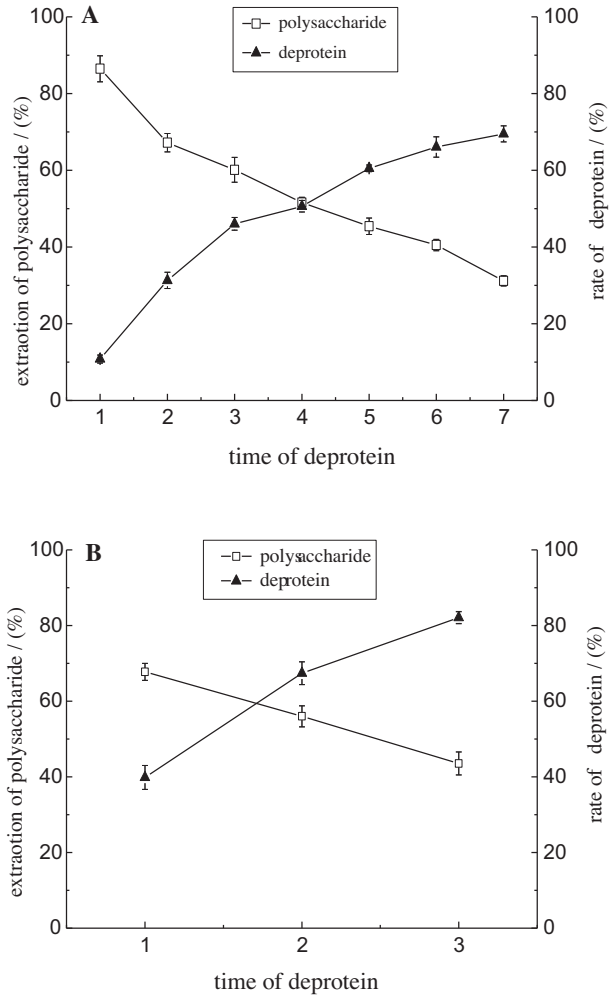

Fig. 1. Deproteinization of the polysaccharides by Sevag method. Crude polysaccharide was mixed with Sevag reagent (chloroform:butanol, 4:1, v/v) at a ratio of 3:1 (v/v) to react at room temperature. (A) W-PTP and (B) A-PTP.

Table 3
Comparison of the physicochemical properties of the two polysaccharides.

Method	W-PTP	A-PTP
Color observation	White	Gray
Solubility test	Hot water soluble, dimethyl sulfoxide soluble	Alkali soluble, weak hot water soluble
Coomassie brilliant blue reaction	(−)[a]	(−)
Ninhydrin reaction	(−)	(−)
Iodination reaction	(−)	(−)
Folin–Wu reaction	(−)	(−)
Benedict reaction	(−)	(−)
$FeCl_3$ reaction	(−)	(−)
Sulfuric acid–carbazole reaction	(+)[b]	(+)
Monosaccharide composition	Glucuronic acid (15.81%), galactose (39.958%), and glucose (44.232%)	Glucuronic acid (35.642%), arabinose (8.820%), galactose (28.467%), and glucose (27.691%)
Total sugar (mg/ml)	0.788 ± 0.033	0.478 ± 0.041
Protein (mg/ml)	0.008 ± 0.001	0.035 ± 0.004
Molecular weight	163,700 Da	305,600 Da

[a] Negative.
[b] Positive.

there was significantly different between in the molecular weight range between the two polysaccharides.

3.6. Conformational analysis

The interaction of W-PTP/A-PTP with Congo red was shown in Fig. 3. The maximum absorption wavelength (λ_{max}) of Congo red was largely shifted in the presence of W-PTP/A-PTP (increased initially and then reduced gradually to reach constant with the increasing NaOH concentration), which indicated that W-PTP/A-PTP had triple-helix conformation, and the triple helical structure was destroyed in high concentrate of NaOH solution (Rout et al., 2008).

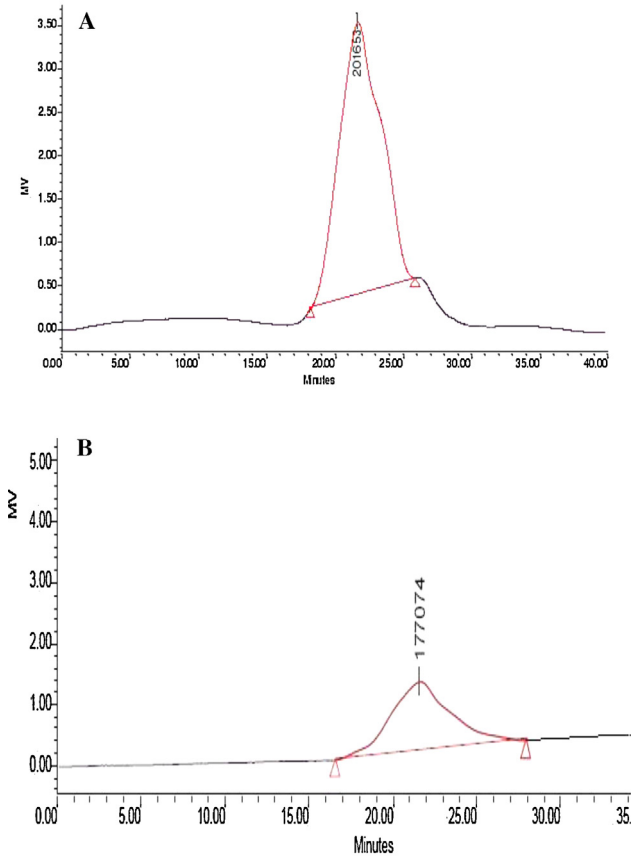

Fig. 2. GPC chromatography of W-PTP and A-PTP based on the calibration curve with standard dextrans. GPC was equipped with two gel columns (TSK PWXL G-5000 and G-3000) coupled with a refractive index detector, eluting with 0.02 mol/l KH_2PO_4 (PH = 6.0) at 0.6 ml/min. (A) W-PTP, the peak with elution volume of 12.972 ml corresponded to M_n of 129,200 Da, its M_w of peak position at 163,700 Da and (B) A-PTP, the peak with elution volume of 12.940 ml corresponded to M_n of 85,200 Da, its M_w of peak position at 305,600 Da.

3.7. Monosaccharide composition

W-PTP/A-PTP was hydrolyzed by TFA into individual monosaccharide, which was further acetylated for GC analysis. Results obtained from GC analysis of W-PTP/A-PTP using a HP-5 capillary column were shown in Fig. 4. According to the retention time of the standard substances and samples, the monosaccharide types could be determined. The content of monosaccharide is calculated by each peak area. A-PTP contained arabinose, while it was not detected in W-PTP. Both A-PTP and W-PTP contain glucuronic acid, galactose, and glucose, but their content in the two polysaccharides exhibited much difference. Sugars present in A-PTP were 27.691, 28.467, 8.820 and 35.642% corresponding to glucose, galactose, arabinose and glucuronic acid in the molar ratio of 3.14:3.23:1:4.04. Sugars present in W-PTP were 44.232, 39.958 and 15.81% corresponding to glucose, galactose and glucuronic acid in the molar ratio of 2.80:2.53:1.00.

Such a difference between the two polysaccharides extracted by two methods was attributed to the different extract solvents. During the extracting process, cell wall prevents intracellular polysaccharide substance dissolution. Alkali solution would be

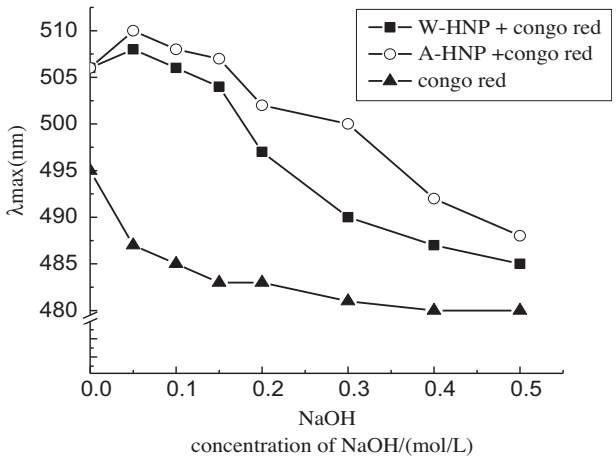

Fig. 3. The maximum absorption wavelength (λ_{max}) of Congo red in the presence of W-PTP and A-PTP at various concentration of sodium hydroxide solution.

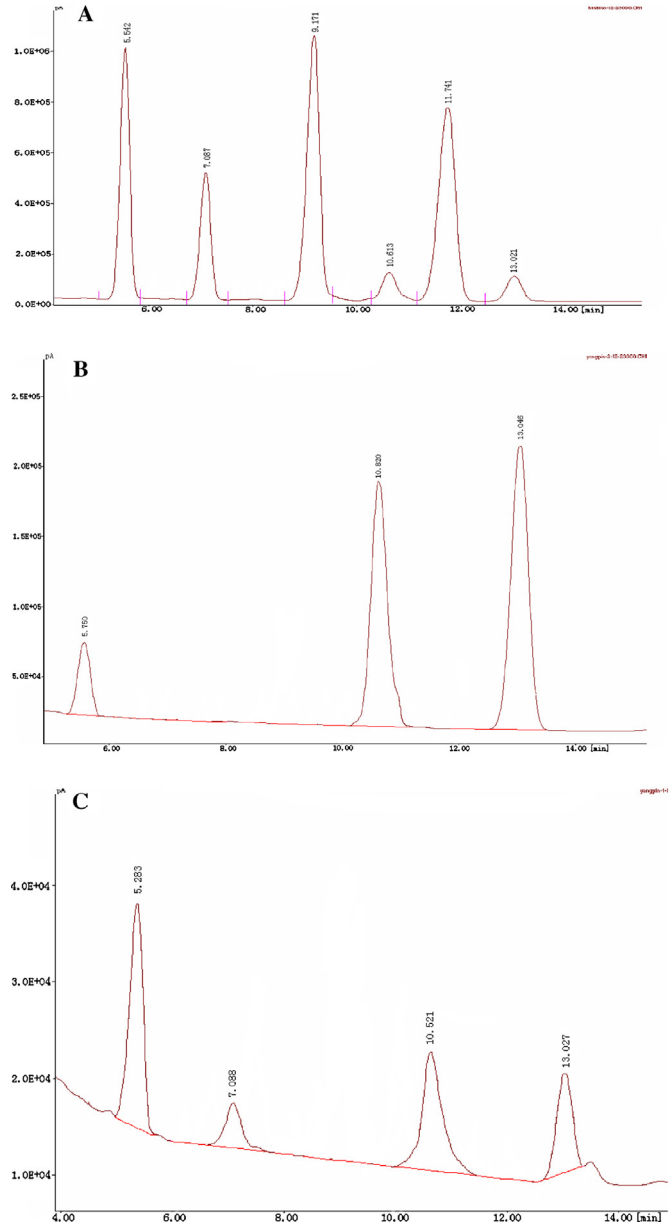

Fig. 4. Gas chromatography of monosaccharide standards, W-PTP and A-PTP from *Pleurotus tuber-regium*. (A) Monosaccharide standards, peak 1 represent for glucuronic acid, 2 for arabinose, 3 for xylose, 4 for galactose, 5 for sorbose and 6 for glucose. (B) W-PTP, peak 1 represent for glucuronic acid (content of 15.81%), peak 2 for galactose (39.958%) and peak 3 for glucose (44.232%). (C) A-PTP, peak 1 represent for glucuronic acid (content of 35.642%), peak 2 for arabinose (8.820%), peak 3 for galactose (28.467%) and peak 4 for glucose (27.691%).

more prone to cell wall damage, thus facilitating intracellular polysaccharide dissolution (Zhao et al., 2012). In addition, solubility is also an important factor. Some water-soluble polysaccharides cannot be dissolved in alkali solution, or alkali-soluble polysaccharides cannot be dissolved in hot water. It led to the different monosaccharide composition of polysaccharides directly. Moreover, those low content polysaccharides may not be detected by HPLC.

Comparing the findings by different authors, we find that the polysaccharide compositions of *P. tuber-regium* have a relatively large difference. Zhang and Cheung (2011) found two components (mannose and glucose) in *P. tuber-regium*. However, Wong et al. (2007) reported three sugar components (glucose, mannose and galactose) in the polysaccharides of the fruiting body of

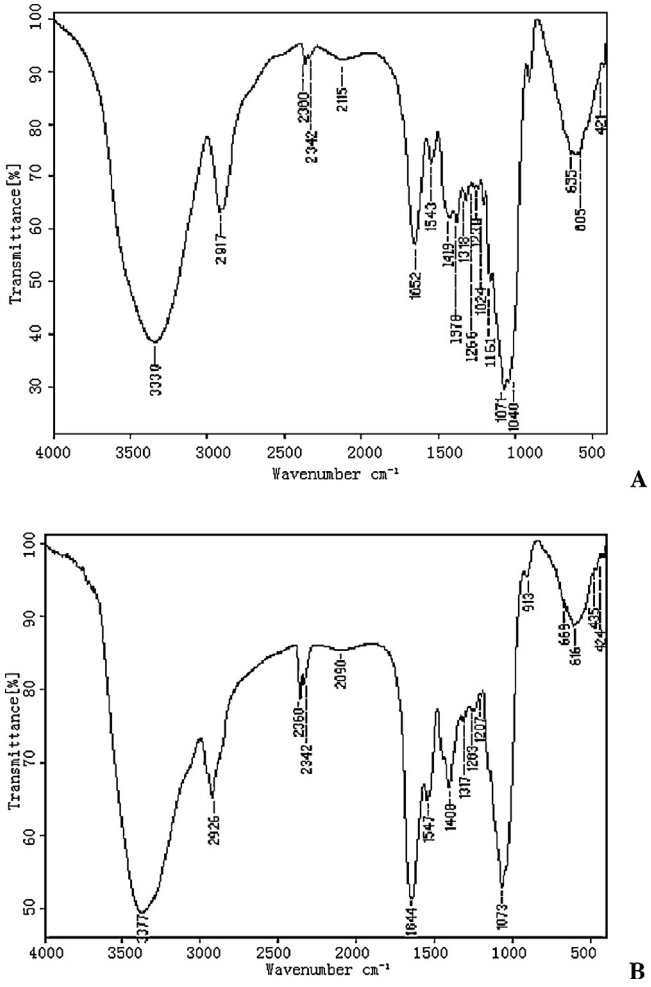

Fig. 5. IR spectrum of the two polysaccharides from *Pleurotus tuber-regium*. All reactions were performed using the KBr-disk method in the range of 4000–500 cm^{-1} at room temperature. (A) W-PTP and (B) A-PTP.

P. tuber-regium, but three sugar components were different from other authors' research (Wong, Cheung, & Wu, 2003). In addition, Zhang et al. (2004) found four components (mannose, glucose, galactose and N-acetyl glucosamine) in the polysaccharides of mycelia and sclerotia of *P. tuber-regium*. These differences may be related to different cultivation regions, analysis methods, and samplings (Zhong, Jin, Lai, Lin, & Jiang, 2010). Different analysis method can also cause different results (Matsuhiro, Lillo, Sáenz, Urzúa, & Zárate, 2006). The polysaccharides constituents are easily oxidized with chemical methods.

3.8. Infrared spectrum

The evidence of sulfate could also be proved by the appearance of the peak at 1230 cm^{-1} (Fig. 5a), which was derived from the stretching vibration of S=O of sulfate. The signals at 3339 and 2917 cm^{-1} were from stretching vibration of O—H and C—H, respectively. In addition, an asymmetrical stretching peak noticed at 1652 cm^{-1} and a weak symmetrical stretching peak near 1400–1200 cm^{-1}, suggested the presence of carboxyl groups (Gnanasambanda & Proctor, 2000).

As shown in Fig. 5b, the IR spectra of A-PTP was displayed a broad and intense peak at around 3377 cm^{-1}, which was assigned to the hydroxyl groups stretching vibration. The weak peak toward 2926 cm^{-1} was attributed to the C—H antisymmetrical stretching vibration. The absorption at 913 cm^{-1} indicated β-pyranose of

glucose (He et al., 2007; Zhao, Kan, Li, & Chen, 2005). The evidence of sulfate could also be proved by the appearance of the peak at 1263 cm^{-1}, which was derived from the stretching vibration of S=O of sulfate (Li et al., 2011).

4. Conclusion

Two polysaccharides, W-PTR and A-PTR from the sclerotia of *P. tuber-regium* were extracted using hot water and alkali methods, respectively. The optimum extraction conditions for the two polysaccharides were obtained by orthogonal experiment methods. The best conditions for W-PTR: extraction temperature 100 °C, solid/liquid ratio 1:30, and extraction time 2 h; for A-PTR: solid/liquid ratio 1:6, NaOH concentration 0.5 mol/l, and extraction time 2 h. W-PTR was composed of glucose, galactose and glucuronic acid with the percentage of 15.81%, 39.958%, 44.232%. A-PTR was composed of glucuronic acid, arabinose, galactose and glucose with the percentage of 35.642%, 8.820%, 28.467%, 27.691%. The differences between the two polysaccharides do exist in color, solubility, molecular weight and monosaccharide composition. The further challenge will be the analysis on the 3D structure of the two polysaccharides, and their comparative biological activity as well as the structure–function relationship.

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