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Purification and identification of one glucan from golden oyster mushroom (*Pleurotus citrinopileatus* (Fr.) Singer)

Jicheng Liu*, Yongxu Sun*, Haitao Yu, Chunjing Zhang, Liling Yue, Xiuzhen Yang, Liping Wang, Jianhua Liu

Qiqihar Medical University, Qiqihar 161006, PR China

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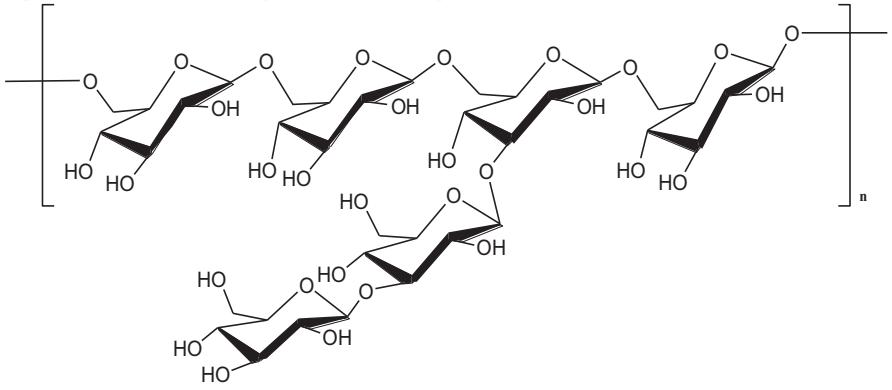
ABSTRACT

One water-soluble glucan (PCP-W1) was purified from the crude polysaccharide of *Pleurotus citrinopileatus* by chromatography on DEAE Sepharose Fast Flow and Sephadex G-200 column, and PCP-W1 (*Mw* = 45 kDa), was predominantly composed of Glc. Partial acid hydrolysis, Smith degradation–periodate oxidation, methylation analysis, Fourier transform infrared spectroscopy (FTIR), gas chromatography–mass spectrometry (GC–MS) analysis and nuclear magnetic resonance spectroscopy (NMR) experiments were conducted to elucidate its structure. The results indicated that PCP-W1 had a glucan backbone consisting of (1 → 6)-linked-β-D-glucopyranosyl residues, which were branched at O-3 position of the backbone with (1 → 3)-linked-β-D-glucopyranosyl and non-reducing end 1-β-D-glucopyranosyl residues. The repeating unit of the polysaccharide was established as:



1. Introduction

Mushrooms have captured the attention of some researcher on account of their exploitable bioactive constituents. Many edible mushrooms, in particular those rich in polysaccharides or polysaccharide–protein complex, not only taste good but also have become an important source of bioactive compounds beneficial to the treatment of various diseases, including hypolipidemic, antioxidant, hypoglycemic, immunostimulant, antitumor activities and so on (Chan, Chan, & Sze, 2009; Sun, Li, & Liu, 2010; Sun, Li, Yang, Liu, & Kennedy, 2010; Sun, 2011). The higher Basidiomycetes species *Pleurotus citrinopileatus*, commonly called "golden oyster mushroom", has recently become a popular delicacy in China, Japan, and Taiwan, for its taste and health value. This attractive species of mushroom in clusters of small and funnel shaped bright-yellow caps grows on fallen trees and stumps of borad-leaf tree species, as shown in Fig. 1. The extract of this mushroom is effective in antioxidation, cardiovascular disease prevention, immunomodulation, antihyperglycemic and antitumor activities (Chen, Ma, Tsai, Wang, & Wu, 2010; Hu et al., 2006). The first information published on the structural features of polysaccharides from the fruiting bodies of *P. citrinopileatus* was reported by Zhang et al. (1994) in Japan. Authors only give the characterization of three

* Corresponding authors.
E-mail addresses: jichengliu@yahoo.cn (J. Liu), yongxusun1978@yahoo.com.cn (Y. Sun).

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Fig. 1. The mushroom of *P. citrinopileatus.*

water-soluble and four water-insoluble polysaccharides, which possessed potent antitumor activity against Sarcoma 180 implanted in mice. Recently, Minato (2008) separated the crude polysaccharides of *P. citrinopileatus* into six fractions (HWE-P-I, II, III, IV, V, and VI) by anion exchange chromatography using DEAE-Sepharose gel, and measured their immunomodulating activity in cytokine production from the stimulated macrophages. The results indicated that HWE-P-IV showed the strongest immunomodulating activity in TNF-α and NO production from stimulated macrophages RAW264. However in the present study we purified one new water-soluble polysaccharide from the fruiting bodies of *P. citrinopileatus*, different from the early reported ones. Therefore in order to better understand the underlying mechanism that this mushroom takes effect to human body, we intend to make a preliminary structural investigation on this polysaccharide.

2. Experimental

2.1. Materials

DEAE Sepharose Fast Flow, Sephadex G-200, and Sephadex G-25 were purchased from Amersham (Sweden). T-series dextran was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

2.2. Isolation and purification of polysaccharide

The dried fruiting bodies of *P. citrinopileatus* was percolated with cold EtOH for 1 week and dried. The residues were extracted 6 times with boiling water (30 L, 6–7 h/per time). After concentration, 4 vol of EtOH was added. The produced precipitate was dried in vacuo at 40 °C. This crude polysaccharide (PCP) was deproteinated by proteinase digestion and the Sevag method (Sun et al., 2008), followed by exhaustive dialysis with water for 48 h. An aliquot was then applied to a column (2.6 cm × 40 cm) packed with DEAE Sepharose Fast Flow, and eluted with stepwise gradient of NaCl aqueous solutions (0, 0.2, 0.4, and 0.6 M) at a flow rate of 4 ml/min, respectively.

The water elution was further purified using Sephadex G 200 gel permeation chromatography (2.6 cm × 100 cm) to give one main purified polysaccharide, code as PCP-W1. All the collection of each tube was monitored by the method of Dubois's at 490 nm, and protein absorption at 280 nm was also measured. Gel permeation chromatography was performed on an ÄKTA explore 100 purification system equipped with a pump P-900, a UV-900 monitor, a pH/C-900 monitor, a Fraction Collector Frac-950, and an autosampler A-900.

2.3. Measurement of carbohydrate and protein contents

Total carbohydrate content of the polysaccharide was determined by phenol–sulfuric acid colorimetric method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). In addition, protein in the polysaccharide was quantified according to the Bradford's method (1976) using bovine serum albumin (BSA) as the standard.

2.4. Homogeneity and molecular weight and the FTIR spectrum analysis

The homogeneity and molecular weight of PCP-W1 was evaluated and determined by high-performance size-exclusion chromatography (HPSEC) (Sun & Liu, 2009). An aliquot of 20 μL the sample solution (0.5%) was performed on a SHIMADZU HPLC system fitted with one TSK-G3000 PW_{XL} column (7.8 mm ID × 30.0 cm), eluted with 0.1 mol/L Na_2SO_4 solution at a flow rate of 0.5 ml/min and detected by a SHIMADZU refractive index detector (RAD-10A). The column was calibrated with T-series dextran (T-130 80, 50, 25, 10) as standards. The molecular weight of PCP-W1 was estimated by reference to the calibration curve made above.

The FTIR spectra (KBr pellets) were recorded on SPECORD in a range of 400–4000 cm^{-1}.

2.5. Analysis of monosaccharide composition

Purified polysaccharide (10 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 3 h. Then the hydrolyzed products were co-evaporated with alcohol to remove the redundant TFA until the PH arrived at about 7. Before KBH_4 reduction for 1.5 h, the hydrolyzed products was adding 0.1 M/L Na_2CO_3 and 1 mg inositol (as internal references) at 30 °C for 50 min, and then was dropwise added 25% acetic acid to neutralize KBH_4 to PH about 6. After desalted by cation exchange resin, filtered by quantitative filter paper and neutralized by evaporating with methanol, the reaction solution was mixed with 1 mL n-propylamine and anhydrous pyridine for 30 min at 55 °C. Finally dried sample was mixed into 0.5 mL anhydrous pyridine and acetic anhydride for 1 h at 90 °C (Jones & Albersheim, 1972; Oades, 1967). The derived acetic esters were analyzed by GC as previously mentioned (Sun & Liu, 2009). Sugar identification was done by comparison with reference sugars (rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, glucuronic acid, and galacturonic acid). The absolute configuration of the monosaccharide was determined according to the method using (+)-2-butanol described by Gerwig, Kamerling, and Vliegenthart (1979).

2.6. Partial acid hydrolysis

PCP-W1 (100 mg) was hydrolyzed with 0.5 M TFA (3 ml) at 90 °C for 5 h. The hydrolyzed PCP-W1 was centrifuged to remove the precipitation, and the supernatant was dialyzed against distilled water for 24 h. Ethanol was added to the solution in dialysis sack after dialyzed, and the precipitation and supernatant were recovered after centrifugation. The fraction out of dialysis sack was collected. All fractions were dried for GC analysis as mentioned above (Sun et al., 2010a, 2010b).

2.7. Periodate oxidation and Smith degradation

PCP-W1 (25 mg) dissolved in 12.5 ml of distilled water was mixed with 12.5 ml of 30 mM $NaIO_4$, and the mixture was kept

in the dark at 4 °C. About Each aliquot (0.1 ml) was withdrawn from the mixture at every 3–6 h intervals and read in a spectrophotometer at 223 nm after it was diluted to 25 mL (Linker, Evans, & Impallomeni, 2001). The reaction was complete when absorbency did not descend, and the excess of $NaIO_4$ was decomposed with ethylene glycol (0.2 ml). Consumption of $NaIO_4$ was measured by a spectrophotometric method (Chaplin & Kennedy, 1994; Dixon & Lipkin, 1954) and the production of formic acid was determined by titration with 0.00458 M NaOH aqueous solution. The reaction mixture was dialyzed in moving tap-water (24 h) and distilled water (24 h), and the retentate was concentrated and reduced with $NaBH_4$, neutralized with 50% acetic acid, and then dialyzed. One-third of the content inside the dialysis sack was lyophilized and analyzed by GC. The rest fraction was hydrolyzed by adding equivalent volume of 1 M H_2SO_4 for 40 h at 25 °C, neutralized to pH 6.0 with $BaCO_3$ and filtered for analysis of Smith degradation. The filtrate was dialyzed, and the content out of sack was desiccated for GC analysis; the content inside was diluted with ethanol, and the supernatant and precipitate were also dried out for GC analysis after centrifugation.

2.8. Methylation analysis

The sample (20 mg) was methylated three times according to the method of Needs and Selvendran (1993). Complete methylation was confirmed by the no absorption peak of the OH band in the region of 3200–3700 cm^{-1} in the FTIR spectrum. The methylated products were hydrolyzed with formic acid and 2 M TFA, then reduced with $NaBH_4$ for 24 h and acetylated with acetic anhydride–pyridine (1:1) at 100 °C for 2 h as described by Sweet, Shapiro, and Albersheim (1975). The alditol acetates of the methylated sugars were analyzed by GC–MS.

2.9. ^1H and ^{13}C NMR analysis

The ^1H and ^{13}C NMR spectra were recorded using a Bruker 5 mm broadband observe probe at 50 °C with a Bruker Advance 500 spectrometer (Germany), operating at 500 MHz for ^1H and 125 MHz for ^{13}C. Deuterium-exchanged PCP-W1 (20 mg) was dissolved in deuteroxide (99.99% D, 0.55 mL), and centrifuged to remove the excessive sample. All the experiments were recorded using standard Bruker software. TMS was used as external standard for the ^{13}C NMR spectrum, and D_2O was used as internal standard for ^1H NMR spectrum.

The above methods applied in this paper are expressed in a conventional way as other papers published by our research group.

3. Results and discussion

3.1. Isolation and purification of polysaccharide

Presently there are many methods for purifying the polysaccharide or protein/peptide bound polysaccharide. They are all labor costing and time consuming. In particular the poor reproducibility of production for these macromolecules have limited their applications in pharmaceutical and food industries. In our research, all chromatography were performed on an ÄKTA explore 100 purification system equipped with a pump P-900, a UV-900 monitor, a pH/C-900 monitor, a Fraction Collector Frac-950, and an autosampler A-900. Various kinds of columns and gel met the demand for purification of polysaccharide from the mushroom. In addition, the operations of data collection were driven by UNICORN software which guaranteed quick, simple communications between systems and users, and can satisfy the stringent control and data handling procedures of modern laboratories (Nie, Xie, Fu, Wan, & Yan, 2008). The result identified that ÄKTA explore 100 purification system was faster, simpler, more effective and reproductive for the purification of PCP-W1 and easier to be extended to the industrial production than the other methods. The PCP-W1 showed a single symmetrically sharp peak, as determined by HPSEC, which indicated PCP-W1 was homogeneous (Fig. 2). The molecular weight of PCP-W1 was estimated to be 45 kDa according to the calibration curve with standard dextrans.

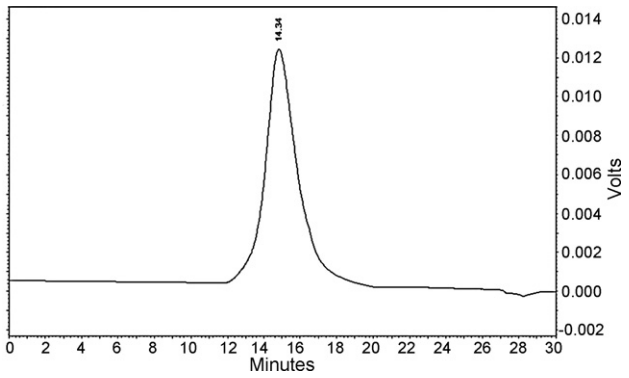

Fig. 2. HPSEC profile of PCP-W1.

Table 1
GC–MS analysis of methylated PCP-W1.

Peak no.	Methylated sugars	Linkage type	Molar ratio
Residue A	2,3,4,-Me_3-Glc*p*	1,6-linked-β-D-Glc*p*	3
Residue B	2,4-Me_2-Glc*p*	1,3,6-linked-β-D-Glc*p*	1
Residue C	2,4,6-Me_3-Glc*p*	1,3-linked-β-D-Glc*p*	1
Residue D	2,3,4,6-Me_4-Glc*p*	1-linked-β-D-Glc*p*	1

3.2. Chemicophysical properties and monosaccharide composition of polysaccharide

The total sugar content of PCP-W1 was determined to be 91.3% using the phenol–sulfuric method. The PCP-W1 was composed of only Glc, as detected by GC (Fig. 3), and the absolute configuration test revealed that all monosaccharides in the glucan are of D configuration. It had a negative response to the Bradford test and no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid.

3.3. Structural analysis of polysaccharide

The FTIR spectrum showed that the strong band appeared at 3432.4 cm^{-1} was attributed to the hydroxyl stretching vibration of the polysaccharide, and 2924.5 cm^{-1} was due to the C–H stretching

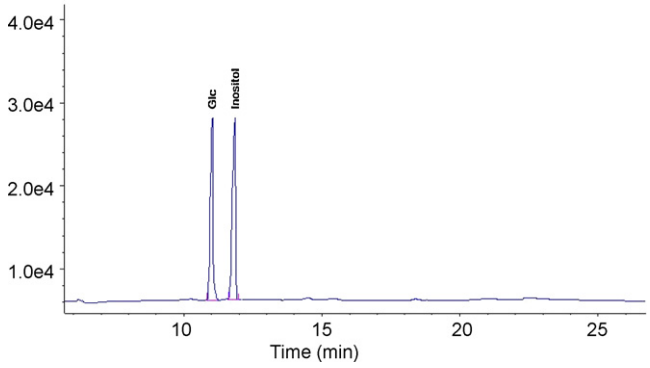

Fig. 3. GC profile of PCP-W1.

Table 2
^{1}H and ^{13}C NMR chemical shift data (δ, ppm) for PCP-W1.

Residue	$^{1}H/^{13}C$						
	1	2	3	4	5	6a	6b
A: →6)-β-D-Glc*p*-(1→	4.53	3.34	3.64	3.51	3.76	3.91	4.18
	103.9	74.1	74.2	70.5	75.3	70.1	
B: →3, 6)-β-D-Glc*p*-(1→	4.53	3.39	3,69	3.58	3.45	3.91	4.18
	103.9	74.1	85.2	69.1	75.8	68.9	
C: →3)-β-D-Glc*p*-(1→	4.82	3.42	3.71	3.58	3.82	3.88	3.79
	103.9	74.3	85.2	69.1	75.7	61.4	
D: β-D-Glc*p*-(1→	4.59	3.41	3.56	3.68	3.52	3.93	3.72
	103.9	75.2	75.2	67.2	74.2	61.4	

vibration absorption. Characteristically, the bands at 1054.6 cm^{-1} suggested the presence of pyranose ring of the glucosyl residue in polysaccharide. Furthermore, the characteristic absorptions at 892.31 cm^{-1} in the IR spectra indicated β-configurations existing in PCP-W1 (data not shown).

Methylation analysis (Table 1) of PCP-W1 mainly produced four partially methylated alditol acetates detected by GC–MS, namely 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl glucitol, 1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl glucitol, 1,3,5-tri-acetyl-2,4,6-tri-O-methyl glucitol and 1,5-diacetyl-2,3,4,6-tetra-O-methyl glucitol. Based on these results, we can concluded that PCP-W1 was mainly composed of 1, 6-linked-β-D-glucopyranosyl (Residue A) and 1, 3, 6-linked-β-D-glucopyranosyl (Residue B) residues as the backbone, and the side chain of 1, 3-linked-β-D-glucopyranosyl (Residue C) as well as non-reducing end 1-linked-β-D-glucopyranosyl (Residue D) residues in the ratio of 3:1:1:1, which was well in agreement with the results of Periodate oxidation–Smith degradation.

The data of ^{1}H NMR and ^{13}C NMR spectra were listed in Table 2. Both a typical β-glycosidic configuration of anomeric carbon observed at δ 103.9 in the ^{13}C NMR spectrum and the corresponding signals δ 4.53, δ 4.53, δ 4.82, δ 4.59 in the ^{1}H NMR spectrum for H-1 of Residues A–D confirmed that all the residues in polysaccharide are of β configuration. Because of C-3 or C-6 was substituted by other sugar residues, C-3 signals of Residues B and C were both downshifted to the high magnetic field with the value of δ 85.2. And same effect happened to C-6 of Residues A and B that downshifted to δ 70.1, δ 68.9 respectively. All signals were identified with respect to those of β-D-glucans reported in the literatures (Carbonero et al., 2006; Han, Chai, Jia, Han, & Tu, 2010; Kruppa et al., 2009; Monteiro et al., 2000; Mondal et al., 2008; Nandan et al., 2008; Vasconcelos et al., 2008).

4. Conclusion

Our data indicated that PCP-W1 from the fruiting bodies of *P. citrinopileatus* possessed the following structure: the backbone consisted of the repeating unit [→6)-β-D-Glc*p*-(1 → 6)-β-D-Glc*p*-(1 → 3,6)-β-D-Glc*p*-(1 → 6)-β-D-Glc*p*-(1→]; the side chain and no-reducing terminal [→1)-β-D-Glc*p*-(3 → 1)-β-D-Glc*p*] was attached to the backbone through O-3 of Glc residues. The further detailed structure elucidation would continue in our later research.

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