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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · MARCH 2014

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Mushroom Dietary Fiber from the Fruiting Body of *Pleurotus tuber-regium*: Fractionation and Structural Elucidation of Nondigestible Cell Wall Components

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ABSTRACT: The cell wall of mushroom fruiting body is constituted of nondigestible macromolecules that are a rich source of dietary fiber with biological functions that are beneficial to human health. The cell wall components of an edible mushroom fruiting body from *Pleurotus tuber-regium* (PTR) were fractionated, and their chemical structures were investigated by chemical, physicochemical, and microscopic analyses. The present results suggest that the cell wall of the PTR mushroom fruiting body contains four main fractions: an outer fraction of polysaccharide and protein complex, which can be extracted using boiling water; a cold alkali-soluble fraction of heteropolysaccharides associated with a small amount of proteins; a hot alkali-soluble fraction of hyper-branched glucans; and an alkali-insoluble fraction of glucan–chitin complex with a normalized relative percentage of 3.6:21.9:55.7:18.8. The anomeric linkage of all the glucans was revealed by infrared spectroscopy to be β type. The structure of the major mushroom fruiting body cell wall polysaccharide (the hot alkali-soluble one, FHA-I) was elucidated by the methylation analysis to be composed of $\rightarrow 1$ -GlcP-(4 \rightarrow linkages as the backbone with a 52% degree of branching consisting of $\rightarrow 1$ -GlcP-(6 \rightarrow linkages in the side chains, whereas some $\rightarrow 1$ -GlcP-(3 \rightarrow linkages might exist in the backbone or side chains. Size exclusion chromatography coupled with multiangle laser light scattering analysis revealed that FHA-I had a molecular weight of 4.224×10^6 g/mol and a root-mean-square radius of 30.4 nm. Both scanning electron and atomic force microscopy further showed the highly branched microstructure of FHA-I when dispersed in an aqueous sodium dodecyl sulfate solution.

KEYWORDS: dietary fiber, mushroom fruiting body, cell wall polysaccharides, *Pleurotus tuber-regium*

INTRODUCTION

Mushroom cell wall (MCW) components (especially the nonstarch polysaccharides) are considered to be a rich source of dietary fiber that has health benefits to humans.¹ The chemical composition of MCW components varies in the different developmental stages of the mushroom life cycle, which includes the mycelium, fruiting body, and sclerotium.^{2,3} Although the composition of MCW is known for some mushroom species with the mycelium and sclerotium in particular,^{3–5} the detailed assembly of individual components in the cell wall, especially those in the fruiting body is still not clear.⁶ The main components of MCW are proteins, chitin, and glucans.^{5–7} In mycelial cell wall, the highly branched β -1,3-/1,6-glucans are reported to position at the cell surface and the linear β -1,3-glucans are linked to crystalline chitin, whereas α -1,3-glucans serve as amorphous matrix throughout the wall.⁷ MCW polysaccharides and polysaccharide–protein complexes are nondigestible biopolymers with potent biological activities including immunopotential, anticancer, and tumor inhibition.^{8,9} Thus, research on the chemical structure of MCW is essential to elucidate the mechanisms of their biological activities and facilitate their applications as dietary fiber.^{10,11}

Pleurotus tuber-regium (PTR) is a basidiomycetes that has a life cycle consisting of mycelium, sclerotium, and fruiting body.^{4,12} β -Glucans and polysaccharide–protein complex extracted from the different morphological stages of PTR have been evaluated as dietary fiber that has various potential

health benefits to humans.^{1,4,8,10,13} In our previous study, the cell wall structure of PTR sclerotium has been elucidated by fractionation and characterization of its soluble polysaccharides.¹⁴ Two sclerotial MCW polysaccharides including a hyper-branched β -glucan, with $\rightarrow 1$ -GlcP-(4 \rightarrow linkages as the backbone with $\rightarrow 1$ -GlcP-(6 \rightarrow linkages in the side chains, and a linear β -glucan with $\rightarrow 1$ -GlcP-(3 \rightarrow linkages were found in sclerotial cell wall. In addition, the structure and conformation of the hyper-branched β -glucans were determined by use of nuclear magnetic resonance (NMR) spectroscopy, transmission electron microscopy (TEM), and atomic force microscopy (AFM).¹⁵ Compared to sclerotium, the cell wall composition and chemical structure of the polysaccharides in the fruiting body of PTR are still not clear. We have found that the content and composition of cell wall proteins in PTR sclerotium and fruiting body were quite different,¹⁶ implying that the MCW components including the polysaccharides in the fruiting body of PTR might also be different from those in the sclerotium. A detailed fractionation and structural characterization of the MCW polysaccharides in the fruiting body of PTR can provide useful information for the preparation of MCW polysaccharides as novel mushroom

Received: January 8, 2014

Revised: March 10, 2014

Accepted: March 13, 2014

Published: March 13, 2014

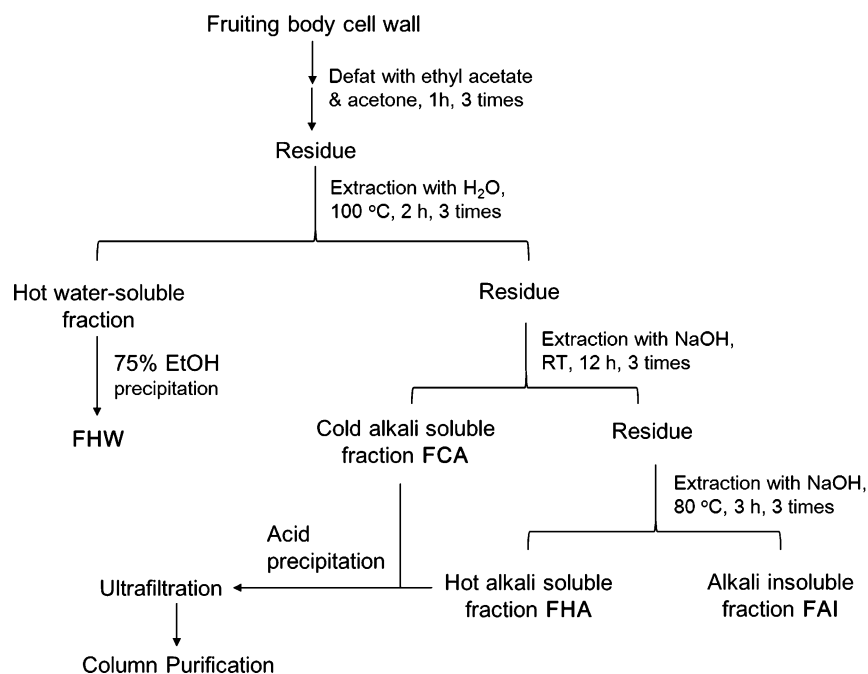


Figure 1. Fractionation scheme of mushroom cell wall of PTR fruiting body.

dietary fiber. This paper focuses on the fractionation of the major soluble MCW polysaccharides from PTR fruiting body and their structural elucidation by use of chemical, spectroscopic, and microscopic techniques.

MATERIALS AND METHODS

Cultivation of Fruiting Body and Preparation of Mushroom Cell Wall. Fruiting body was obtained from the incubation of PTR sclerotium embedded in soil kept in a greenhouse under humid and warm conditions (30–35 °C) for 2 months. The harvested fruiting body was freeze-dried and stored at −20 °C before use. Freeze-dried sample of fruiting body was pulverized into powders to pass through a screen with an aperture of 0.5 mm by using a cyclotech mill (Tecator, Höganäs, Sweden). The milled powders were suspended in distilled water and ultrasonicated at 300 W with a 30% duty cycle for 10 min (Sonics & Materials Vibracell, model VC 600) for cell breakage. The degree of cell breakage was monitored by a phase contrast microscope (PCM, Eclipse 80i, Nikon). Cleaved mushroom cells were washed sequentially with wash solution A [1 mM phenylmethanesulfonyl fluoride (PMSF)], wash solution B [5% (w/v) NaCl, 1 mM PMSF], wash solution C [2% (w/v) NaCl, 1 mM PMSF], wash solution D [1% (w/v) NaCl, 1 mM PMSF], and then wash solution A again to remove intracellular contaminants.¹⁶ Each step was repeated three times until no carbohydrate and protein could be determined from the washed buffer using the phenol–sulfuric acid method and bicinchoninic acid (BCA) protein assay,^{17,18} respectively. The isolated MCW was lyophilized prior to its fractionation.

Fractionation and Purification of MCW from PTR Fruiting Body. MCW of PTR fruiting body was fractionated according to the scheme shown in Figure 1. After the successive extraction using hot water, cold alkali, and hot alkali solution (1 M NaOH), three soluble MCW fractions including the hot water-soluble fraction (FWH), the cold alkali-soluble fraction (FCA), and the hot alkali-soluble fraction (FHA) were obtained from the supernatants after centrifugation (4000g for 10 min at 4 °C), leaving behind an alkali-insoluble residue (FAI). The FWH fraction was concentrated and subjected to ethanol fractional precipitation up to 75% ethanol. The supernatants of the two alkali-soluble fractions FCA and FHA were precipitated using 75% ethanol to precipitate the polysaccharides and then washed five times using 75% ethanol until the washings were neutral. FAI was washed with Milli-Q water five times to remove any residual chemicals.

FCA and FHA were subjected to further fractionation by membrane ultrafiltration (Millipore, stirred ultrafiltration 8040) with molecular weight (MW) cutoff of 10000 Da to give two fractions (FCA-I and -II; FHA-I and -II) with MW >10000 and <10000 Da, respectively. As larger MW fractions, FCA-I and FHA-I were further purified by gel filtration on a Sephacryl S-1000 Superfine column combined with a Sephacryl S-400 high-resolution column (90 × 2.6 cm i.d., Amersham Pharmacia Biotech, UK) eluted with 0.2 M NaCl at 1.5 mL/min. Being smaller MW fractions, FCA-II and FHA-II were further purified to a single fraction by a Sephacryl S-200 high-resolution column (70 × 2.6 cm i.d., Amersham Pharmacia Biotech, UK) eluted with 0.2 M NaCl at 1.5 mL/min. All of these purified fractions were desalted by gel filtration on a Sephadex G10 column (70 × 2.6 cm i.d.; Amersham Pharmacia Biotech, UK) eluted with distilled water and then freeze-dried prior to further analyses.

Chemical Composition Analysis. The carbohydrate content of the MCW was estimated by using the phenol–sulfuric acid method,¹⁷ whereas the uronic acid was determined colorimetrically according to the *m*-hydroxydiphenyl–sulfuric acid method.¹⁹ Protein content was analyzed using the bicinchoninic acid (BCA) protein assay as described previously.¹⁸

The monosaccharide composition of the purified polysaccharides from the MCW of PTR fruiting body was determined by the alditol acetate derivatives of the sugars after acid hydrolysis, reduction, and acetylation as described previously²⁰ using a gas chromatograph (GC) (6890N, Agilent Technology, USA) fitted with an Alltech DB-225 capillary column (15 m × 0.25 mm i.d., 0.25 μm film) and coupled with a mass spectrometer (MS) (5973N, Agilent Technology, USA). The oven temperature program were as follows: initial temperature, 170 °C; temperature raised at 2 °C/min to 220 °C, and final hold for 15 min. Helium was used as carrier gas at a flow rate of 1.0 mL/min. The injector temperature and interface temperature were set at 250 and 280 °C, respectively. The MS conditions were as follows: ion source temperature at 250 °C, ionization energy at 70 eV, detector voltage at 1.5 kV, and mass range from 50 to 400.²⁰ Individual sugars were corrected for losses during hydrolysis and derivatization as well as their different responses to the MS detector.²⁰

Linkage Analysis by Methylation. The purified polysaccharides from fruiting body cell wall material (FCWM) were permethylated to partially methylated alditol acetates (PMAA) using methyl iodide and solid NaOH in dry DMSO as described in a modified protocol reported previously.²¹ The PMAA samples were then analyzed by GC-

MS with an Alltech DB-225 capillary column (15 m \times 0.25 mm i.d., 0.25 μ m film) as mentioned above. The oven temperature was increased from 130 to 220 $^{\circ}$ C by 4 $^{\circ}$ C/min. The MS detector conditions were the same as mentioned above. Each PMAA was identified by matching its mass spectrum with the literature database.²²

Molecular Mass and Size. The molecular parameters (including molecular mass and size) of purified polysaccharide were determined using a size exclusion chromatograph (SEC) (Agilent 1200 series LC system) coupled with a multiangle laser light scattering (MALLS) photometer (DAWN EOS, Wyatt Technology Co., USA) and refractive index detector (RI, G1362A, Agilent Technologies Inc., USA). The purified soluble MCW polysaccharide fractions were dissolved in 0.15 M NaCl to a concentration of 1.0 mg/mL and filtered through a 0.22 μ m nylon syringe filter before determination. A serial column that combined TSK G-5000 (30 cm \times 7.5 mm i.d., Supelco, USA) with TSK G-3000 columns was then employed to separate the samples at 30 $^{\circ}$ C. The predegassed 0.15 M NaCl aqueous solution was applied as the elution buffer at a flow rate of 0.5 mL/min. A specific refractive index increment (dn/dc) value of the polysaccharides in 0.15 M NaCl aqueous solution was determined with a differential refractometer (OPTILAB DSP) (λ = 690 nm) to be 0.140 mL/g. Astra software (Wyatt Technology Co.) was utilized for data recording and further analysis.

Infrared Spectrum Analysis. The infrared spectra of the purified MCW polysaccharides, hot water-soluble fractions, and alkali-insoluble fractions from MCW of PTR fruiting body were recorded with a Fourier transform infrared spectrometer (FT-IR, Nicolet 670) in the range of 4000–400 cm^{-1} using a KBr disk method.

Microscopic Analysis. The protocols used were according to the previous analysis of MCW polysaccharides from PTR sclerotium¹⁶ and hyper-branched glucans.¹⁴ To observe the microstructure of a single molecule, sodium dodecyl sulfate (SDS) (used as a small molecular surfactant to disperse the entangled molecules) was added to a 1 mg/mL FHA-I aqueous solution at a ratio of 1:1 (w/w) and heated together at 80 $^{\circ}$ C for 2 h. The solution was diluted stepwise to a final concentration of 5 μ g/mL and was heated to 80 $^{\circ}$ C for 2 h with constant stirring.

The holey carbon film (200 mesh, Beijing Zhongjingkeyi Technology, China), supported by a copper grid, was used to prepare the sample for the TEM viewing. After filtration through a 0.22 μ m nylon syringe filter, a droplet of the sample (5 μ g/mL) was deposited on the specimen, which was finally dried in air at ambient temperature and humidity. The molecular morphology of the prepared samples was performed on a TEM (H-7650, Hitachi, Japan) at an accelerating voltage of 80 kV.

To observe the AFM image, 5 μ L of filtered sample solution (5 μ g/mL) was pipetted briefly onto the freshly cleaved surface of a mica disc (Ted Pella, Inc., USA). The surface was air-dried for 20 min in a covered Petri dish and then washed with a large amount of distilled water before fixing with ethanol. After the surface was dried under the same condition as the TEM sample, it was examined using an AFM (Nanoscope III, Veeco Instruments Inc., USA) in the tapping mode, which was equipped with a Si probe (Tap 150-G-10, Ted Pella, Inc., USA) with a quoted spring constant of 1.5–15 N/m at a cantilever driving frequency of 300 kHz and a scanning frequency of 1–2 Hz. Nanoscope software was used to obtain and process AFM images.

RESULTS AND DISCUSSION

Cell Wall Content and Composition. The cell wall content of PTR fruiting body was determined to be $46.6 \pm 0.9\%$ dry weight (DW), which was significantly less than that from sclerotium ($78.4 \pm 0.7\%$ DW) according to our previous analysis of sclerotial cell wall.¹⁴ In general, PTR fruiting body MCW contained 74.6% carbohydrate, 4.27% uronic acids, and 3.27% protein (Table 1). In contrast, the chemical composition of the mycelial cell wall was found to contain 79.0% carbohydrates, 5.1% uronic acids, and 3.8% proteins, whereas

Table 1. Composition of Fractions from Fruiting Body Cell Wall^a

sample	% DW	total carbohydrate (%)	uronic acid (%)	protein (%)
FHW	3.37 ± 0.13	42.7 ± 0.4	0.22 ± 0.04	49.4 ± 0.61
FCA	20.5 ± 0.16	90.1 ± 0.4	0.76 ± 0.07	8.18 ± 0.38
FHA	52.4 ± 0.77	98.5 ± 0.8		
FAI	17.6 ± 0.54	72.9 ± 0.4	5.96 ± 0.23	
MCW		74.6 ± 0.8	4.27 ± 0.18	3.27 ± 0.03

^aData are the mean \pm SD (n = 3).

that of sclerotial cell wall contained 71.6% carbohydrates, 1.6% uronic acids, and 7.0% proteins (unpublished data).

Four crude fractions (FHW, FCA, FHA, and FAI) were obtained from the MCW of PTR fruiting body, and their chemical compositions are shown in Table 1. Hot-water-soluble fraction (FHW) accounted for only 3.4% DW of the MCW of PTR fruiting body, whereas the two alkali-soluble fractions constituted 72.9% of the MCW (20.5 and 52.4% for FCA and FHA, respectively). The relative percentage of these two alkali-soluble fractions (FCA and FHA) was similar to that of mycelial cell wall (22.0 and 47.0% for mycelial cold-alkali and hot-alkali fractions, respectively) but was different from sclerotial cell wall with the main component being the cold alkali-soluble fraction (41.2 and 35.3% for sclerotial cold-alkali and hot-alkali fractions, respectively).¹⁴ FAI constituted 17.6% of MCW, which was similar to that from mycelial and sclerotial alkali-insoluble cell residues (19.0 and 16.9%, respectively) (unpublished data). FHW had a very high level of protein (49.4% by weight), indicating the existence of proteins or glycoproteins in the outer layer of the MCW of PTR fruiting body. A relatively lower level of proteins (8.18%) found in the FCA fraction suggested the existence of proteins in the middle layer of the MCW of PTR fruiting body. Levels of uronic acids were very low among the MCW fractions except for FAI. The alkali-soluble fractions (FCA and FHA) of the PTR fruiting body were predominantly composed of carbohydrates, whereas the FAI fraction contained 72.9% carbohydrates, which was higher compared with that in mycelial and sclerotial alkali-insoluble residues (45.7 and 57.4%, respectively) (unpublished data).

On the basis of the fractionation, the arrangement of the cell wall components in PTR fruiting body from outer layer to inner layer was suggested to be in the following order: proteins or glycoproteins, polysaccharides, complex of polysaccharide and chitin.

Purification of Polysaccharides from PTR Fruiting Body MCW. Other than FHW, which had little amount, FCA and FHA were purified by using membrane filtration and column chromatography. After purification by use of membrane filtration, fractions with larger molecular weight (MW > 10000) were obtained and then further purified using column chromatography to give a single fraction (FCA-I and FHA-I). Fractions with smaller MW (<10000) (FCA-II and FHA-II) obtained from membrane separation were then subjected to column chromatography purification, but very little amount of samples could be recovered (<7% of total fraction). Therefore, the analysis of purified MCW polysaccharides was mainly focused on two high MW soluble fractions (FCA-I and FHA-I).

Monosaccharide Composition of Polysaccharides from PTR Fruiting Body MCW. The monosaccharide composition of FHW was mainly glucose with small amounts

Table 2. Chemical Composition of Cell Wall Polysaccharide Fractions from PTR Fruiting Body^a

sample	monosaccharides (%)				glucosamine ^b	total carbohydrate (%)	uronic acid (%)
	xylose	mannose	galactose	glucose			
FHW	3.19	19.64	3.45	73.72		42.2	0.22
FCA-I		22.22	11.24	66.54		92.1	0.77
FHA-I				100.00		97.4	
FAI				72.23	27.77	72.9	5.96

^aData are the means of triplicates. ^bGlucosamine is derived from chitin

of mannose, galactose, and xylose as shown in Table 2. Compared with FHW, the cold alkali-soluble polysaccharide (FCA-I) contained more mannose and galactose, but without xylose. After purification, protein was not detected in FCA-I, although 8.17% protein was found in FCA. FHA-I as the main fraction of PTR fruiting body MCW was composed of almost purely glucose (Table 2). Different from the other soluble MCW fractions, FAI contained 72.2% glucose [similar to the total carbohydrate content of 72.9% (Table 1)], whereas 27.3% of this fraction was composed of glucosamine (the structural subunit of chitin). FAI was thus composed of an insoluble glucan–chitin complex that is commonly found in fungi.

Glycosidic Linkages of FHA-I. Due to the poor solubility of FCA-I and FAI in DMSO, linkage analysis was performed only on FHA-I. PMAAs in the GC-MS chromatogram of FHA-I were identified by their retention time in the total ion chromatogram (TIC) and by comparison with mass spectrum patterns from the literature database.²² The glycosidic linkages and their corresponding percentages are listed in Table 3. The molar ratio of monosaccharide residues was calculated according to the peak areas and response factors for individual sugars in the TIC as mentioned above.

Table 3. Peak Area and Molar Ratio of Glycosidic Linkages in FHA-I by Methylation Analysis^a

linkage pattern	retention time (min)	FHA-I	
		peak area (%)	molar ratio
T-Glcp ^b	13.999	26.61	5.64
1,3-Glcp	16.991	5.13	1.00
1,6-Glcp	17.972	10.93	2.13
1,4-Glcp	18.170	32.05	6.25
1,3,6-Glcp	21.097	8.19	1.48
1,4,6-Glcp	21.319	17.09	3.08
TU/BP ^c		1.05	
DB ^d			0.52

^aData are the means of triplicates. ^bGlcp, glucopyranose. ^cTU/BP, terminal units/branching points. ^dDB = (NT + NB)/(NT + NB + NL), the degree of branching, where NT, NB, and NL are the numbers of the terminal residues, branch residues, and linear residues, respectively.

Consistent with the results of polysaccharides from PTR sclerotium MCW,¹⁴ FHA-I consisted of a nonreducing terminal glucose, 1,3-D-Glcp, 1,4-D-Glcp, 1,6-D-Glcp, 1,3,6-D-Glcp, and 1,4,6-D-Glcp. The molar ratios of these six sugar residues were similar to those polysaccharides from PTR sclerotium MCW.¹⁴ However, the percentages of 1,4-D-Glcp and 1,3,6-D-Glcp in FHA-I had obviously increased, whereas residues of T-D-Glcp and 1,4,6-D-Glcp had decreased. The major glycosidic linkage in FHA-I was 1,4-D-Glcp residues (32.1%), followed by T-D-Glcp (26.6%) (Table 3). The major branched sugar residues in FHA-I were 1,4,6-D-Glcp, which accounted for 17.1% of the total

sugar residues, whereas 8.19% of 1,3,6-D-Glcp existed in this fraction. The ratio between terminal units (T-D-Glcp) and branching points (1,4,6-D-Glcp and 1,3,6-D-Glcp) was 1.05 (TU/BP ratio) (Table 3). Consistent with the structure of polysaccharides from PTR sclerotium MCW,¹⁴ the data from the above methylation analysis suggested that FHA-I was also mainly composed of →1)-D-Glcp-(4→ residues as the backbone with some →1)-D-Glcp-(6→ residues in the side chains, whereas some →1)-D-Glcp-(3→ residues might exist in the backbone or side chains. The major branching points of FHA-I were at O-4 and/or O-6 positions of the Glcp chain. The degree of branching (DB) of FHA-I was calculated by the equation [DB = (N_T + N_B)/(N_T + N_B + N_L)] to be 52%. With such a high DB value, FHA-I would be likely to be a hyper-branched glucan consistent with polysaccharides purified from PTR sclerotium MCW.¹⁴

Molecular Mass and Size of FHA-I. The SEC chromatogram of FHA-I indicated that there was only a very small shoulder peak (<9% total peak area) present in front of the major peak (Figure 2) that might be caused by the aggregation of the polysaccharide. The values of M_w , M_n , root-mean-square (RMS) radius/radius of gyration ($\langle S^2 \rangle^{1/2}$) of the samples were obtained by computing a classical Zimm plot from light-scattering data (data not shown).²³ The d_f value can be determined from the relationship between M_w and RMS radius and ν according to eqs 1 and 2, known as the inverse power law functions.²⁴

$$\text{RMS radius} = kM_w^\nu \quad (1)$$

$$d_f = 1/\nu \quad (2)$$

From the slope of the angular dependence (Figure 3), the M_w of the FHA-I was estimated to be 4.224×10^6 g/mol, whereas its RMS radius was calculated to be 30.4 nm (Table 4). FHA-I, which was purified from FHA, accounted for 52.4% DW of the PTR fruiting body MCW, indicating that this fraction is the major structural skeleton in the cell wall. The value of M_w/M_n is known as the polydispersity index, which reflects MW homogeneity and a value of 1 for monodispersed molecules.²⁵ The value of M_w/M_n (1.089) for FHA-I (Table 4) indicated the relatively high purity of FHA-I.

According to previous studies, the exponent ν in eq 2 reflects the molecular shape of a polymer with values of 0.33, 0.50–0.60, and 1.0 representing a sphere, random coil, and rigid rod, respectively.^{26,27} The small RMS radius of FHA-I (30.4 nm) (Table 4) suggested that this macromolecule might exist as a compact conformation in aqueous solution.^{28,29} The ν value of FHA-I was found to be 0.33, and its corresponding d_f value was 3.06 (Table 4), indicating that the conformation of the FHA-I was spherical.^{26,27,30} As described in previous studies,^{31–33} this was consistent with the highly branched structure suggested by the above linkage analysis (Table 3).

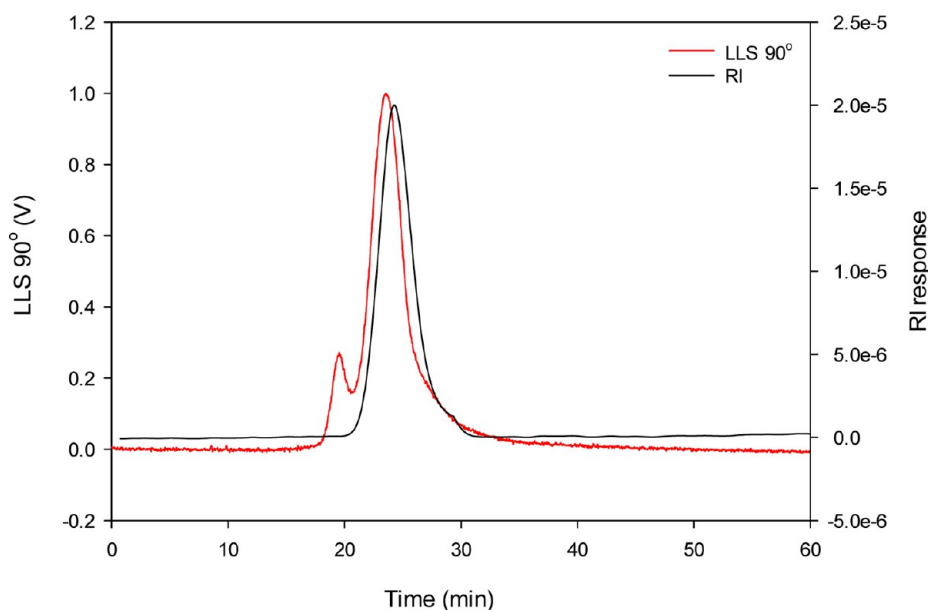


Figure 2. SEC-MALLS chromatograms of FHA-I.

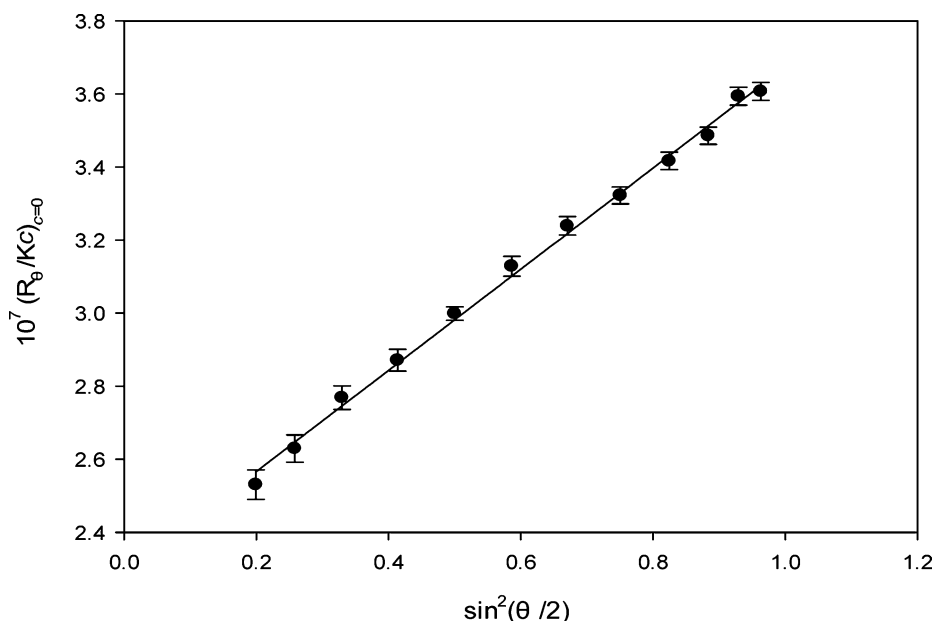


Figure 3. Angular dependence of $(R_{\theta}/Kc)_{c=0}$ for FHA-I, where K is the optical constant, c is the concentration, R_{θ} is the Rayleigh ratio, and θ is the scattering angle.

Table 4. Results of SEC-MALLS for FHA-I^a

sample	$M_w \times 10^{-6}$ (g/mol)	RMS radius (nm)	M_w/M_n	d_f	ν
FHA-I	4.224 ± 0.77	30.4 ± 4.9	1.089	3.06	0.33 ± 0.08

^aData are the mean \pm SD. M_w , weight-average molecular mass; M_n , number-average molecular mass; d_f , fractal dimension; ν , exponent in eq 1.

IR Spectrum of Polysaccharides from PTR Fruiting Body of MCW. All of the IR spectra of the four fractions from MCW (Figure 4) exhibited an absorption peak at ~ 900 cm^{-1} , which was similar to the characteristic absorption peak for the β configuration of glucan (890 cm^{-1}).³⁴ The absorption peak at 834 cm^{-1} of FCA-I indicated the existence of an α configuration of glucan. The unique absorption of double

peaks at 1661 and 1629 cm^{-1} combined with the peak at 1565 cm^{-1} in the spectrum of FAI indicated that a secondary amide group ($-\text{CO}-\text{NH}-\text{C}$) existed in this fraction due to the presence of a large amount of chitin and was consistent with the monosaccharide composition data (Table 2) for a glucan–chitin complex.

Molecular Morphology of FHA-I. To further observe the microstructure of the spherical FHA-I, SDS was added to break the hydrogen bonds inside the sphere. Both branched conformations and crossed chains were observed under this condition (Figure 5). Figure 5A shows part of a particular FHA-I molecule with an extended conformation. Similar to the hyper-branched PTR sclerotium MCW cold alkali-soluble fraction (SCA-I),¹⁵ a high degree of branching was observed from the TEM image of FHA-I (Figure 5A) with a large

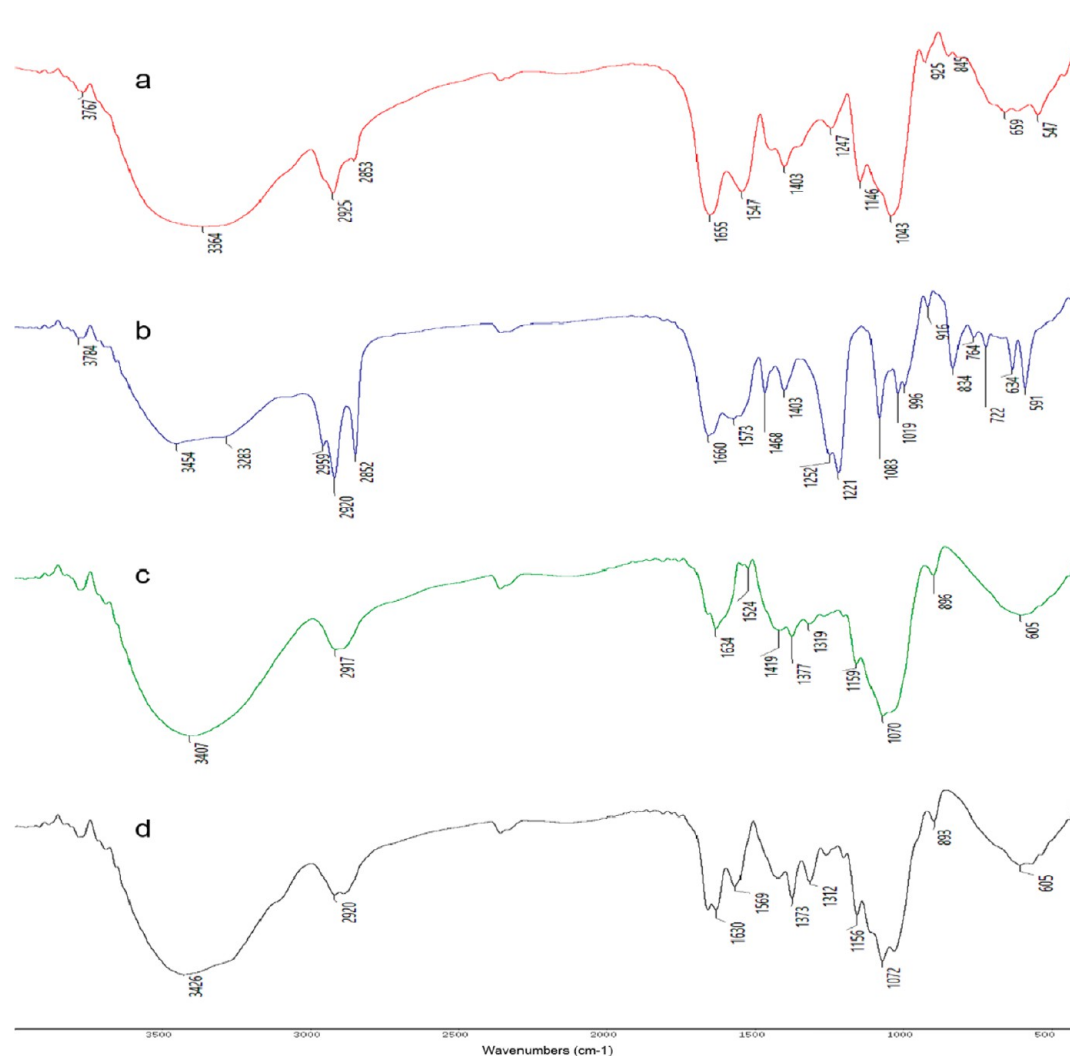


Figure 4. IR spectrum of polysaccharide fractions derived from mushroom cell wall of PTR fruiting body: (a) FHW; (b) FCA-I; (c) FHA-I; (d) FAI.

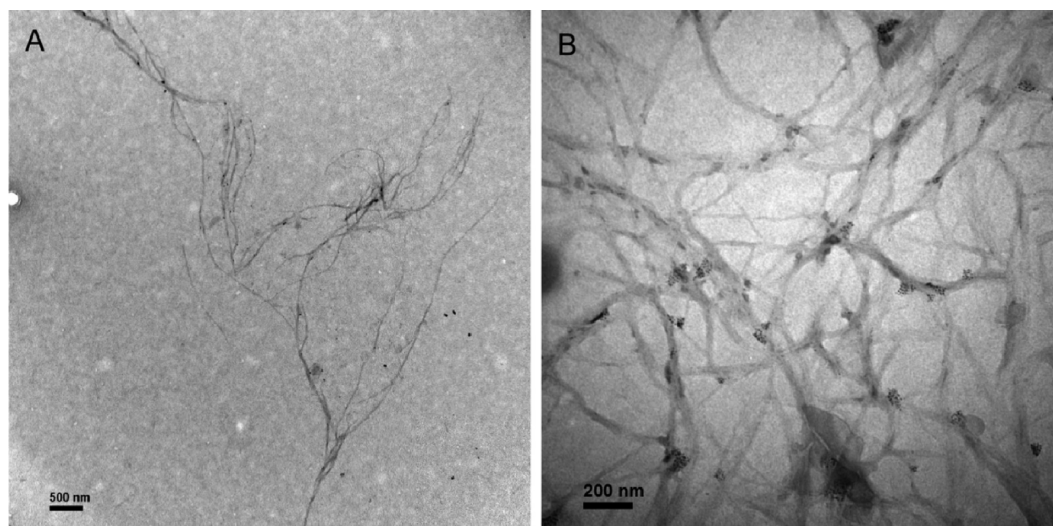


Figure 5. TEM images of FHA-I in SDS solution: (A) unfolded conformation; (B) entangled chains.

number of the polymer chains entangled together in the fully extended state shown in Figure 5B.

Branched conformation of FHA-I molecule in SDS could also be found in AFM (Figure 6A), which was similar to the

result in TEM (Figure 5). Furthermore, the thickness of the molecules (indicated by the red pointers in the cross-section line in Figure 6B) varied from 4.8 to 40.4 nm, indicating that multiple chains of FHA-I molecules rather than a single-chain

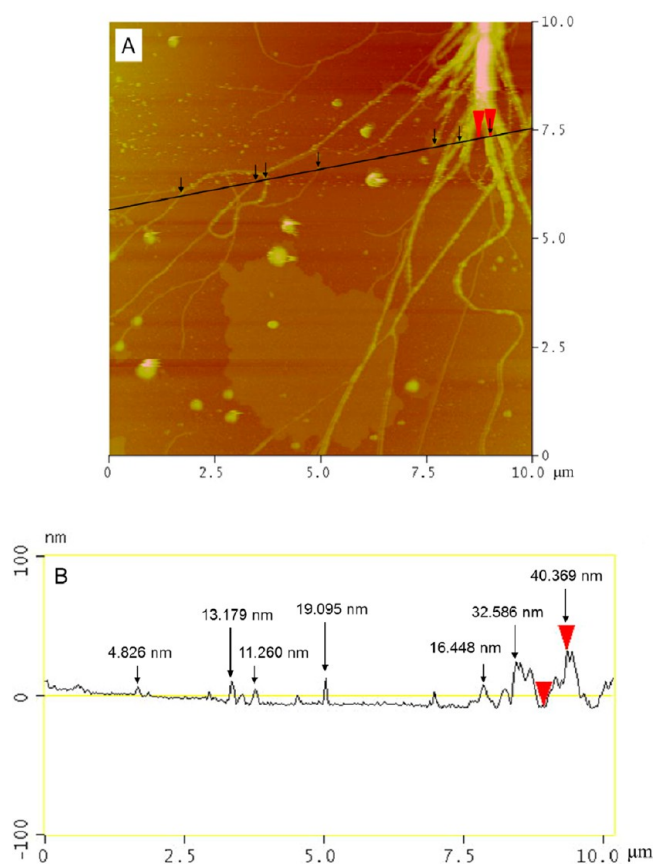


Figure 6. Tapping mode AFM images (A) and cross-sectional profile (B) of FHA-I dispersed in 80 °C SDS solution.

one existed in the SDS medium. With such thickness, it was possible that up to eight chains of macromolecules might be stacked up or bundled together to give such a dimension.

Mushroom Cell Wall Structure of PTR Fruiting Body. A sandwich model was proposed to represent the amount and chemical nature of the cell wall components sequentially extracted from the MCW of PTR fruiting body (Figure 7). Apart from proteins, a small amount of glycan–protein complexes could be found in the first fraction of MCW

(Table 1), all of which could be linked with other cell wall components via noncovalent bonding because these proteins and glycan–protein complexes could be extracted using boiling water.¹⁶ The second fraction of MCW is mainly composed of cold alkali-soluble heteropolysaccharides with glucose, mannose, and galactose (Table 2) with small amounts of proteins (Table 1), suggesting that the glycans are associated with proteins via noncovalent linkage because these proteins could be removed in the purification procedure. The third fraction of MCW could be considered as the main structural skeleton due to its abundance, and it is composed of hyper-branched glucans (Table 3 and Figure 6). The MCW polysaccharides in this fraction could be removed using alkali solution at 80 °C. The innermost fraction of MCW could be composed of glucan–chitin complexes, which contain >70% glucan and <30% chitin (Table 2). This protective barrier could not be removed by hot alkali solution. The normalized relative percentage of the above four fractions in MCW was found to be 3.6:21.9:55.7:18.8 from outer layer to inner layer on the basis of the results in Table 1.

The cell wall structure of other mushroom fruiting bodies has not been studied in detail, but some cell wall polysaccharides have been derived and characterized.^{35,36} A cold alkali-soluble polysaccharide has been purified from the fruiting body of *Laetiporus sulphureus* (Bull.: Fr.) Murr and was named latiglucan I, which was characterized to be a linear β -1,3-glucan (molecular weight = 1.8×10^5 Da) constituting 42.7% by weight of the fruiting body.³⁶ The structure of some polysaccharides obtained from the fruiting body of *Pleurotus* species other than PTR has also been investigated. For instance, the main MCW polysaccharides of the fruiting body of *P. ostreatus* and *P. eryngii* were branched β -1,3-/1,6-glucan and linear α -1,3-glucan, respectively.⁶ Furthermore, the fruiting body of *P. florida* contained two different types of glucans: a water-soluble one consisting of a main chain of α -1,3-linked D-glucan partially substituted at O-3 and O-6 by β -D-glucose³⁷ and a water-insoluble β -1,3-/1,6-D-glucan.³⁸ The structure and degree of branching (DB) of MCW polysaccharide from PTR fruiting body therefore are unique as compared with those found previously in other *Pleurotus* species.

Despite the fact that mushroom cell wall is composed of a water-soluble fraction, an alkali-soluble fraction, and an alkali-insoluble fraction that are similar to the cell wall in lower fungi,

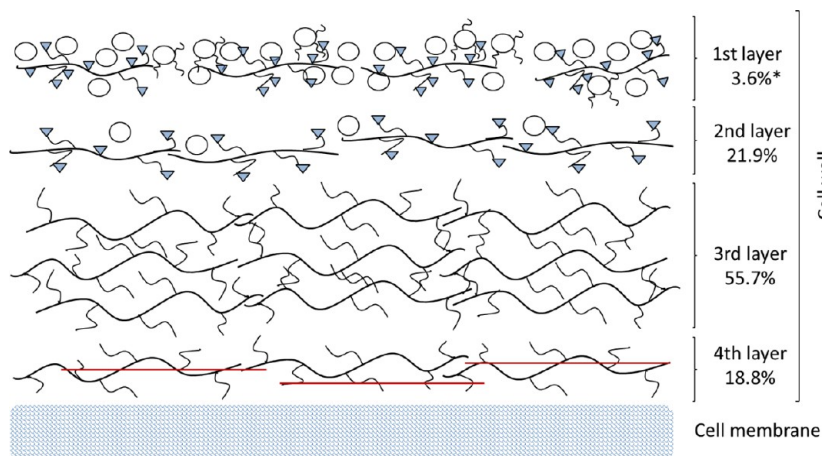


Figure 7. Proposed cell wall structure of PTR fruiting body. (*) The normalized relative percentage of individual cell wall fractions is not shown in an absolute scale. Symbol key: (open circles) protein; (open circles with “legs”) glycoprotein; (lines with “legs”) glucan; (red line) chitin; (blue triangles) other sugar residues (including mannose, galactose, and xylose).

the structure of mushroom cell wall of PTR fruiting body is more complicated than and very different from the cell wall of lower fungi in terms of the chemical fine structure of the cell wall polysaccharides.^{39–41} Such differences could mainly be found in the alkali-soluble fractions. This fraction has been characterized to be β -glucans in the cell wall of yeast and filamentous fungi, which is mainly composed of a long β -1,3 main chain or short β -1,6-linked side chains.^{42–45} However, many more β -1,4 residues was found in the alkali-soluble fractions of MCW in PTR fruiting body. β -1,4-Glucose is commonly recognized as the sugar residue of structural polysaccharides of plant cell wall, such as cellulose.^{46–48} More mannose, galactose, and xylose were found in the alkali-soluble cell wall polysaccharides from the fruiting body in PTR. Moreover, differing from the linear structure of β -glucans found in the cell wall of lower fungi, the alkali-soluble fractions from MCW of PTR fruiting body are mainly hyper-branched polymer with a relatively higher DB (52%) than that of lower fungal cell wall polysaccharides mainly composed of linear β -1,3-/1,4-glucan with 4% of β -1,6 branch points.⁴² The innermost fraction of MCW is composed of glucan–chitin complexes, which account for only <20% by weight. The relative thickness of this fraction in MCW of PTR fruiting body is therefore thinner than that of lower fungi, which accounts for about 40% of their cell wall.⁴²

In summary, the fruiting body of PTR has 47% MCW by dry weight, of which the main components (total 78% of MCW) are the alkali-soluble fractions (FCA and FHA), and this was in contrast with the cell wall of filamentous fungi and yeast. The major fraction of FCWM in PTR is hyper-branched β -1,4-glucans with 52% DB on the O-6 position as compared to the linear β -1,3-glucans with very few branches on the O-6 position found in the cell wall of lower fungi. These results provide some new insights in the study of the structural organization of fungal cell wall, especially for higher fungi. Mushroom fruiting body of PTR thus represents a potential source of novel dietary fiber with unique structural features that might have potential applications in the food industry. Furthermore, research on the biological functions and physiological activities of the fractionated cell wall components of FCWM from PTR is currently underway.

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Funding

This project was financially supported by the Research Committee of CUHK Directed Grant for Research (No. 2030435).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The technical support provided by technicians from the School of Life Sciences and the Department of Physics of CUHK for AFM, SEM, and HPSEC-LLS was gratefully acknowledged.

ABBREVIATIONS USED

AFM, atomic force microscopy; BCA, bicinechonic acid; DB, degree of branching; DW, dry weight; FAI, alkali-insoluble residue; FCA, cold alkali-soluble fraction; FCWM, fruiting body

cell wall material; FHA, hot alkali-soluble fraction; FHW, hot water-soluble fraction; GC, gas chromatograph; MALLS, multiangle laser light scattering; MCW, mushroom cell wall; MS, mass spectrometry; MW, molecular weight; NMR, nuclear magnetic resonance; PCM, phase contrast microscope; PMAA, partially methylated alditol acetates; PMSF, phenylmethane-sulfonyl fluoride; PTR, *Pleurotus tuber-regium*; RI, refractive index; RMS, root-mean-square; SDS, sodium dodecyl sulfate; SEC, size exclusion chromatograph; TEM, transmission electron microscopy; TIC, total ion chromatogram; TU/BP, terminal units/branching points

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