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Structure of a water-soluble heteropolysaccharide from fruiting bodies of *Hericium erinaceus*

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ABSTRACT

A new heteropolysaccharide (HEPF4), with a molecular weight of 2.03×10^4 Da as determined by high-performance liquid chromatography (HPLC), was obtained from the fruiting bodies of *Hericium erinaceus*. It is composed of 3-*O*-methylrhamnose, L-fucose, D-galactose and D-glucose in the ratio of 0.12:1.00:3.27:0.28. Its chemical structure was characterized by sugar and methylation analysis, along with ^{1}H and ^{13}C NMR spectroscopy, including NOESY and HMBC experiments for linkage and sequence analysis. The polysaccharide is composed of a tetrasaccharide repeating unit (described in the results section). HEPF4 also contains a minor proportion of glucose and 3-*O*-methylrhamnose which is believed to terminate the polymer main chain.

1. Introduction

Mushroom-derived polysaccharides have emerged as an important class of bioactive substances, to which numerous medicinal and therapeutic properties have been attributed. *H. erinaceus* (*Hericium erinaceus*) is a traditional Chinese medicinal fungus. It has been used to treat gastric ulcers, chronic gastritis and other digestive tract-related diseases. It has been reported that both the fruiting bodies and the fungal mycelia contain bioactive polysaccharides which are reputed to exhibit various pharmacological activities including enhancement of the immune system, antitumor, hypoglycemic and anti-ageing properties (Nie & Zhu, 2003; Yang, Yan, Wang, & Bai, 2000; Zhou, Liu, Chen, & Wang, 1991). In order to identify the correlations between the structure and functionality, we have conducted structural studies on polysaccharides from *H. erinaceus*. In this paper, the structure of HEPF4, a novel neutral polysaccharide purified from the fruiting body of *H. erinaceus*, was investigated.

2. Experimental

2.1. Materials

Fruiting bodies were purchased from Pan'an in Zhejiang Province, China and identified by Professor Taihui Li from institute of Microbiology of Guangzhou, China. DEAE-Sepharose Fast Flow and Sephacryl S-300,S-400 High Resolution were purchased from Amersham Pharmacia Biotech Dextrans and the monosaccharides, D-Gal, D-Ara, L-Fuc, L-Rha, D-man, D-Xyl and D-Glc, were from Sigma. All other reagents (A.R. grade) were purchased from (the corporation, China). HPLC was carried out on a waters 2695 HPLC system (2695 HPLC Pump, 2414 Refractive Index Detector). GC–MS was carried out using a ThermoFinnigan TRACE MS, and NMR spectra were determined with a Varian INOVA 500.

2.2. Isolation and purification

The total fruiting bodies of *H. erinaceus* were first exhaustively extracted with EtOH under reflux for 12 h to remove lipids. After filtration, the residue was air dried and extracted 3 times with boiling distilled water (2 h for each). The combined aqueous filtrate was concentrated to one-tenth of the original volume. The final alcohol concentration of the aqueous filtrate was adjusted to be 30% with 95% ethanol. The precipitate was separated out, and defined as HEPF30. A portion of HEPF30 was dissolved in water and the insoluble residue was removed by centrifugation (10,000 rpm, 10 min,

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4 °C). The supernatant was applied to a DEAE-Sepharose Fast Flow column (XK 26 cm × 100 cm), eluted with a 0–2 M gradient of NaCl solution. The fractions were collected by a fraction collector and the compounds were determined by means of the phenol-sulfuric acid assay (Zhang, 1999). HEPF30-A was obtained from the water elute. HEPF30-A2 purified by gel permeation chromatography on a column of Sephacryl S-400 High Resolution (XK26 × 100 cm). HEPF4 was purified by a column of Sephacryl S-300 High Resolution (XK26 × 100 cm) from the water elute of HEPF30-A2, whose molecular weight range was detected first on a linked column SN of TSK PWXL 4000 and 3000 gel filtration columns firstly.

2.3. Determination of purity and molecular weight

Homogeneity and molecular weight of samples was determined by HPLC on a linked column of TSK PWXL 4000 and 3000 gel filtration columns, eluting with 0.1 M phosphate buffer solution (PBS) and 0.3 M $NaNO_3$ at pH 7.0 with a flow rate of 0.6 mL/min. The column was kept at 30.0 ± 0.1 °C. The linear regression was calibrated by dextrans (T-700, 580, 300, 110, 80, 70, 40, 9.3, 4). All samples were prepared as 0.2% (w/v) solutions, and 10 μL of solution was injected in each run.

2.4. Sugar analysis

HEPF4 (2 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 110 °C for 2 h, and the monosaccharide composition was determined by high-performance anion-exchange chromatography (HPAEC) using a Dionex LC30 equipped with a CarboPac™ PA20 column (3 mm × 150 mm). The column was eluted with 2 mM NaOH (0.45 mL/min) and the monosaccharides were monitored using a pulsed amperometric detector (Dionex) (Yang, Zhang, &Tang, 2005).

2.5. Methylation analysis

Vacuum dried polysaccharide (2 mg) was dissolved in DMSO (2 mL) and methylated by treatment with NaOH-DMSO (0.2 mL) suspension and methyliodide (0.2 mL) according to the method of Kalyan and Paul (1992). The reaction mixture was extracted with trichloromethane, and the trichloromethane was then removed by evaporation. Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm^{-1}) in the infrared spectrum. The permethylated polysaccharide was hydrolyzed by treatment with formic acid (88%, 0.5 mL), H_2O (0.1 mL) and trifluoroacetic acid (0.05 mL) for 16 h at 100 °C. The partially methylated sugars in the hydrolysate were reacted with sodium borohydride and acetylated by acetic anhydride, and the resulting mixture of alditol acetates was analyzed by GC–MS. and analyzed by GC–MS using a DB-5 column (30 m × 0.25 mm × 0.25 μm) and a temperature program consisting of 80–200 °C at 5 °C/min, increasing to 215 °C at 2 °C/min, and finally to 270 °C at 20 °C/min.

2.6. NMR analysis

HEPF4 (30 mg) was lyophilized three times in D_2O (0.5 mL). The ^1H NMR and ^{13}C NMR (60 °C) spectra were determined in 5 mm tubes using a Varian INOVA 500 NMR spectrometer. ^1H chemical shifts were referenced to the water resonance at δ 4.41 at 60 °C as internal standard. ^{13}C chemical shifts were determined in relation to DSS (δ 0.00 ppm) calibrated externally. DEPT-135 spectroscopy, ^1H–^1H correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY) and heteronuclear multiple quantum correlation spectroscopy (HMQC) was used to assign signals. Two-dimensional heteronuclear multiple-bond correlation spectroscopy (HMBC) and two-dimensional Overhauser effect spectroscopy (NOESY) were used to assign inter-residue linkages and sequences.

3. Results

HEPF4 was purified by anion-exchange and gel filtration chromatography (Fig. 1.) from the fruiting bodies of *H. erinaceus*. The single symmetrical peak presented by HPLC indicated that it was a homogeneous polysaccharide. Based on the calibration curve of dextran standards, the molecular weight of HEPF4 was calculated to be 2.03 × 10^4 Da.

HEPF4 was a white powder which was soluble in water. Lack of absorption at 280 and 260 nm by UV scanning indicated that the polysaccharide did not contain phenolic compounds, protein or nucleic acid. HEPF4 was composed of 3-*O*-rhamnose (Zhang, Zhang, Tang, & Jia, 2006), L-fucose, D-galactose and D-glucose in the ratio of 0.12:1.00:3.27:0.28 by sugar compositional analysis.

The interglycosidic linkages between monosaccharide residues of HEPF4 were investigated by methylation analysis (Table 1). The polysaccharide was methylated twice and after acid hydrolysis, methylated sugars were converted to partially methylated alditol acetates. As a result of methylation analysis, sugar residues consist of 2,3,4-tri-*O*-methylrhamnose, 2,3,4-tri-*O*-methylfucose, 2,3,4-tri-*O*-methylgalacose, 3,4-di-*O*-methylgalacose and 2,3,4,6-tetra-*O*-methylglucose (Fig. 2).

The ^1H NMR spectrum (Fig. 3) of the polysaccharide mainly contained signals for three anomeric protons at δ 5.04–5.13, one CH_3–C group at δ 1.29 ($J_{5,6}$ 4.5 Hz) corresponded to the chemical shift of H-6 of Fuc, with other sugar protons appearing in the region of δ 3.50–4.24, a signal for an *O*-methyl group at δ 3.50, validated by HMQC. The three low-field signals (**a–c**) with the *galacto* configuration all appear as singlets ($J_{1,2}$ < 3 Hz) and represent sugars having the α-configuration (Harding, Marshall, Hernandez, & Maqsood, 2005). The DEPT-135 ^{13}C NMR spectrum of the polysaccharide contained signals for three anomeric carbons at δ 100.8–104.1 and one CH_3–C groups (C-6 of Fuc) at δ 18.4. Sugar ring carbons linked to oxygen in the region of δ 64.15–80.5. The reverse peaks at δ 69.4 and 70.05 were assigned to *O*-substituted C-6 of galactosyl residues and a small reverse peak at δ 64.15 should be unsubstituted C-6 of terminal glucosyl residues. As judged by the absence from the ^{13}C NMR spectrum of signals within δ 82–88, all sugar residues are in the pyranose form (Bushmarinov, Ovchinnikova, Kocharova, & Blaszczyk, 2004).

The ^1H resonances for H-1, 2, 3 of residue **a** were assigned from the cross-peaks in the ^1H–^1H COSY spectrum. The assignment of H-4 was based on the TOCSY spectrum. H-5 and H-6 were assigned from the ^1H–^1H COSY spectrum. The cross-peaks of H-3 and H-6

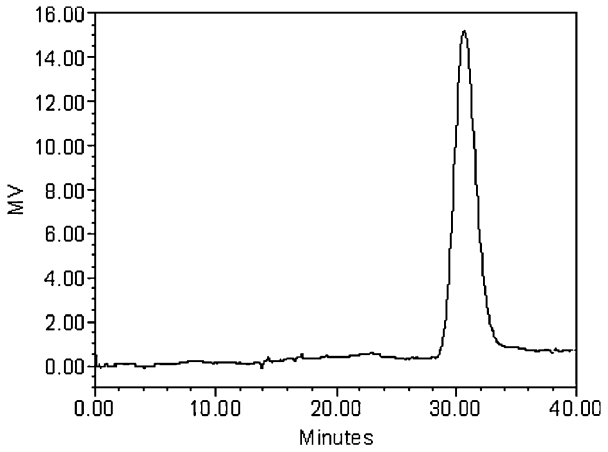

Fig. 1. The HPLC result of HEPF4 fromthe fruiting bodies of *H. erinaceus*.

Table 1
GC–MS data for methylation analysis of HEPF4 isolated from the fruiting bodies of *H. erinaceus*.

Methylated sugar	Type of linkage	Molar ratio	Major mass fragments (*m*/*z*)
2,3,4-Me_3-Rha*p*	1-Linked -*O*-Me-Rha*p*	0.13	43,71,89,101,117,131,145,161
2,3,4-Me_3-Fuc*p*	1-Linked Fuc*p*	1.00	43,72,89,101,115,117,131,161,175
2,3,4-Me_3-Gal*p*	1,6-Linked Gal*p*	2.12	43,71,87,101,117,129,161,173,189,233
3,4-Me_2-Gal*p*	1,2,6-Linked Gal*p*	1.07	43,71,87,99,129,159,173,189,233
2,3,4,6-Me_4-Glc*p*	1-Linked Glc*p*	0.24	43, 71, 87,101,117,129,145,161,205

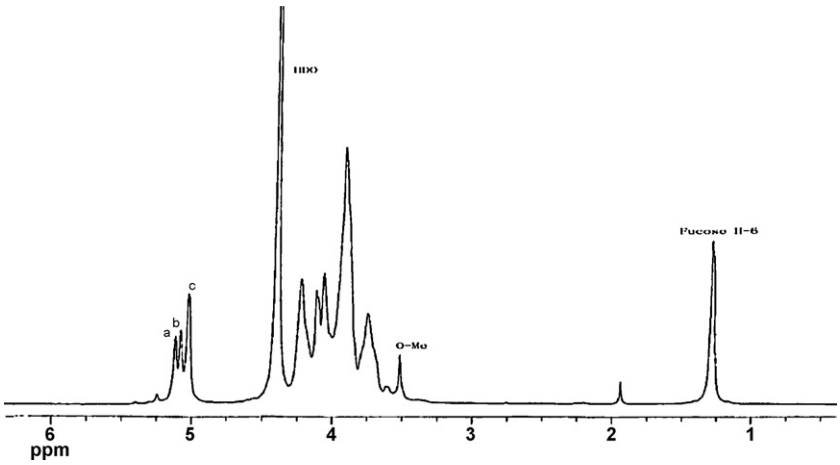

Fig. 2. ^{1}H NMR spectrum of the HEPF4 isolated from the fruiting bodies of *H. erinaceus*in D_2O at 60 °C.

in the NOESY spectrum, H-6 and C-3 in HMBC spectrum, showed that H-5 and H-6 were located on the residue **a**. On the basis of the proton assignments, the chemical shifts of C-1 to C-6 were readily obtained from the HMQC spectrum. Both the carbon and proton chemical shifts were typical of 6-deoxyhexopyranose, and residue **a** could be fucose only since this sugar was the only deoxyglycose identified by GC–MS analysis. Moreover, appears of H-1as a singlet ($J_{H\text{-}1,H\text{-}2} < 3$ Hz) in the ^{1}H NMR spectrum, and H-1/H-2 intra-residue correlations in the NOESY spectrum, provided further support for an α-configuration at the anomeric center. Thus, residue **a** was identified as α-L-fucopyranoside.

^{1}H resonances for H-1, H-2, H-3 and H-4 of residue **b** were assigned from the cross-peaks in the ^{1}H–^{1}H COSY and TOCSY spectra. H-5, H-6a and H-6b were assigned from TOCSY. In the HMBC spectrum, the crosspeaks of H-1 and C-3, C-5 showed that H-5 and H-6 were located on residue **b**. The corresponding ^{13}C resonances were assigned from HMQC. The H-4/5 coupling constant was small, as expected for a Gal-type residue (Staaf, Urbina, Weintraub, & Widmalml, 1999). Residue **b** had an α-configuration at its anomeric center, which was evident from the singlet of H-1 as well as the characteristic $J_{H\text{-}1,H\text{-}2} < 3$ Hz, H-1/H-2 intra-residue correlations in the NOESY spectrum (Stroop, Xu, Retzlaff, Abeygunawardana, & Bush, 2001). The linkage positions were determined from the downfield displacements of the C-2 and C-6 signals. Hence, residue **b** was idenfied as 2,6-disubstituted α-D-galactopyranoside.

Using similar approaches, The TOCSY spectrum demonstrated correlations between the H-1 and all protons from H-1 to H-4 for all sugar residues. The ^{1}H–^{1}H COSY spectrum showed most of the correlations between the neighbouring protons within each spin system, but there were no H-4 and H-5 correlations. The H-5 resonance was assigned from the H-3/H-4 and H-4/H-5 cross-peaks in the NOESY spectrum (Reddy et al., 1998). The H-5, H-6a and H-6b resonances were then obtained from TOCSY specturm. ^{13}C resonances were assigned from the HMQC spectrum. H-4 displayed strong NOEs to both H-3 and H-5, which indicated that residue **c** was a Gal-type residue. H-1 appeared as a singlet ($J_{H\text{-}1,H\text{-}2} < 3$ Hz) in the ^{1}H NMR spectrum and H-1/H-2 intra-residue correlations in the NOESY spectrum indicated that residue **c** had an α-configuration.

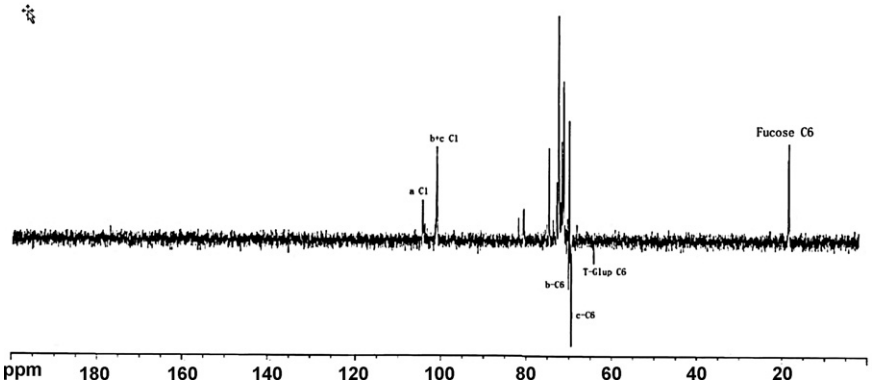

Fig. 3. ^{13}C-DEPT-135 NMR spectrum of HEPF4 isolated from the fruiting bodies of *H. erinaceus*.

Table 2
Chemical shift data for HEPF4 isolated from the fruiting bodies of *H. erinaceus*.

Residue		Proton or carbon						
		1	2	3	4	5	6a	6b
α-L-Fuc*p* (**a**)	H	5.13	3.87	3.93	3.85	4.21	1.29	
	C	104.1	71.3	69.9	71.5	72.3	18.4	
→2,6)-α-D-Gal*p* (**b**)	H	5.09	3.90	4.12	4.23	4.19	3.72	4.03
	C	100.9	80.5	71.3	69.9	72.4	70.05	
→6)-α-D-Gal*p* (**c**)	H	5.04	3.91	4.07	3.92	4.24	3.75	3.98
	C	100.8	74.6	72.4	72.3	72.1	69.4	

The linkage position was assigned from the high chemical shift value for C-6. The remaining residues were assigned to a 6-substituted α-D-galactopyranoside.

By comparison of the chemical shift data (Table 2) for residues **a–c** with those reported for glycosides (Agrawal, 1992) permitted identification of residue **a** as 1-linked α-L-Fuc*p*, residue **b** as 2,6-linked α-D-Gal*p* and residue **c** as 6-linked α-D-Gal*p*.

Once the ^{1}H and ^{13}C NMR spectra had been virtually completely assigned, the sequence of the glycosyl residues in the tetrasaccharide was determined by the observed inter-residue connectivities generated by NOESY and HMBC experiments. Inter-residue NOEs connectivities were observed between H-1 of residue **a** and H-2 of residue **b**, between H-1 of residue **b** and H-6a of residue **c**, and between H-1of residue **c** and H-6a and H-6b of residue **c**. HMBC spectrum showed clear correlations between H-1 of residue **a** and C-2 of residue **b**, between H-1 of residue **b** and C-6 of residue **c**, and between H-1of residue **c** and C-6 of residue **b**.

The results demonstrate that the polysaccharide consists of tetrasaccharide repeating unit with the following structure:

α-L-Fuc*p*
1
↓
2
→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→

HEPF4 also contains a minor proportion of glucose and 3-*O*-methylrhamnose that is believed to terminate the polymer main chain.

4. Discussion

Polysaccharides consisting of a (1 → 6)-linked α-D-galactan backbone and branches composed of glucose and rhamnose have been found in the fungus, *H. erinaceus* (Jia, Liu, Dong, & Fang, 2004). HEPF1 which has a (1 → 6)-linked α-D-galactopyranosyl backbone with branches that are composed of fucose attached to *O*-2 had been isolated from *H. erinaceus*, it also contains 6-*O*-substituted-β-D-oligoglucosyl units and a minor terminal 3-*O*-methyl rhamnose residue (Zhang, Sun, Zhang, & Tang, 2007). Moreover, HEPF3 which has a (1 → 6)-linked α-D-galactopyranosyl backbone with branches that are composed of fucose attached to *O*-2, as well as 3-*O*-methylrhamnose that terminates the main chain, had been characterized in our previous investigation (Zhang et al., 2006). The extraction, isolation and structure characterization of HEPF4 investigated in this report provide a theoretic background and material basis for the further research of polysaccharides from *H. erinaceus*.

Acknowledgement

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