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Structural investigation of a novel fucoglucogalactan isolated from the fruiting bodies of the fungus *Hericium erinaceus*

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Abstract

A new heteropolysaccharide with a molecular weight of 1.94×10^4 Da, HEPF1, was isolated from the fruiting bodies of *Hericium erinaceus*. It is composed of fucose, galactose and glucose in the ratio of 1:4:1, as well as a minor proportion of 3-*O*-methyl rhamnose. Sugar analyses, methylation analysis, together with ^{1}H and ^{13}C NMR spectroscopy established that HEPF1 has a (1 → 6)-linked α-D-galactopyranosyl backbone with branches that are composed of fucose attached to O-2; it also contains 6-*O*-substituted-β-D-oligoglucosyl units and a minor terminal 3-*O*-methyl rhamnose residue.

Keywords: Fucoglucogalactan; *Hericium erinaceus*; Polysaccharide; Structural analysis

1. Introduction

Hericium erinaceus is a traditional Chinese medicinal fungus. Distributed throughout China, it is used to treat gastric ulcers, chronic gastritis and other digestive tract-related diseases. Both the fruiting bodies and the fungal mycelia have been reported to contain bioactive polysaccharides that are reputed to exhibit various pharmacological activities including enhancement of the immune system, and antitumor, hypoglycemic and anti-ageing properties (Nie & Zhu, 2003; Yang, Yan, Wang, & Bai, 2000; Zhou, Liu, Chen, & Wang, 1991). In order to identify correlations between structure and functionality, we have conducted structural studies on polysaccharides from *Hericium erinaceus*. In this paper, the structural investigation of HEPF1, a novel neutral polysaccharide purified from the fruiting body of *H. erinaceus*, is described.

2. Materials and methods

2.1. Materials

Fruiting bodies were purchased from Qing'an in Zhejiang Province, China. DEAE–Sepharose Fast Flow and Sephacryl S-300 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 were of A.R. grade and made in 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

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remove lipids. This step was repeated three times. After filtration, the residue was dried in air at room temperature, and then extracted with boiling distilled water thrice (2 h for each); the liquid extracts were combined. The total aqueous filtrate was concentrated into one-tenth of the original volume, and 95% ethanol was added to the aqueous filtrate until the final alcohol concentration reached 60%. The precipitate was separated out, and defined as HEPF60. A portion of HEPF60 was dissolved in water and the insoluble residue was removed by centrifugation. The supernatant was applied to a DEAE–Sepharose Fast Flow column (XK 26 × 100 cm), eluted first with water and then with a 0–2 M gradient of NaCl. The fractions were collected by a fraction collector and compounds were detected by means of the phenol–sulfuric acid assay, HEPF60-A was obtained by elution with water. HEPF1 was purified by HEP60-A by gel-permeation chromatography on a column of Sephacryl S–300 High Resolution (XK 26 × 100 cm) from HEPF60-A, whose molecular weight range was detected first on a linked column SN of TSK PWXL 4000 and 3000 gel filtration columns.

2.3. Determination of purity and molecular weight

Determination of homogeneity and molecular weight of samples was done 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 column was calibrated by dextrans (T-700, 580, 300, 110, 80, 70, 40, 9.3, 4) using linear regression. All samples were prepared as 0.2 % (w/v) solutions, and 10 μl of solution was analyzed in each run.

2.4. Sugar analysis

HEPF1 (2 mg) was hydrolyzed in 4 ml of 2 M trifluoroacetic acid (TFA) at 110 °C for 2 h. The monosaccharides were conventionally converted into the alditol acetates. 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 °C to 200 °C at 5 °C/min, increasing to 215 °C at 2 °C/min, and finally to 280 °C at 20 °C/min.

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 iodomethane (0.2 ml) by the method (Kalyan & Paul, 1992). The reaction mixture was extracted with $CHCl_3$, and the solvent was then removed by evaporation. Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm^{-1}) in the IR. The permethylated polysaccharide was hydrolyzed by treatment with HCO_2H (88%, 0.5 ml), H_2O (0.1 ml) and CF_3CO_2H acid (0.05 ml) for 16 h at 100 °C. The partially methylated sugars in the hydrolysate were reacted with $NaBH_4$ and acetylated by AC_2O, and the resulting mixture of alditol acetates was analyzed by GC–MS.

2.6. NMR analysis

HEPF1 (30 mg) was lyophilized three times in D_2O (0.5 ml). The ^1H NMR (25 °C, 60 °C) and ^{13}C NMR (25 °C) spectra were determined in 5-mm tubes using a Varian INOVA 500 NMR spectrometer. ^1H chemical shifts were referenced to residual HDO at δ 4.78 ppm (25 °C) as internal standard. ^{13}C chemical shifts were determined in relation to DSS (δ 0.00 ppm) calibrated externally. ^1H–^1H correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY) and heteronuclear multiple quantum coherence (HMQC) were 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 and discussion

HEPF1 was eluted as a single symmetrical peak corresponding to an average molecular weight of 1.94 × 10^4 Da as determined by the HPLC method. Lack of absorption at 280 nm indicated that HEPF1 contained no protein.

The composition of HEPF1 determined by TLC and GC–MS as alditol acetate indicated that HEPF1 consists of L-Fuc, D-Gal, D-Glu, in the molar ratio of 1:4:1, as well as a small amount of 3-*O*-methylrhamnose, which was confirmed by comparison of retention times (Fig. 1, 22.58 min) and mass spectra (Fig. 2) of partially *O*-methylated derivatives. The mass spectrum of 3-*O*-methylrhamnose is dominated by the cleavage of bonds between *O*-methylated carbons and adjacent *O*-acetylated carbons. For 3-*O*-methylrhamnose alditol acetate, this breakage produced *m*/*z* 203 and 189 as the primary fragments, and *m*/*z* 87, 101, 129 and 143 as the other main fragments. This is in agreement with a previously published report (Fox, Black, Fox, & Rostovtseva, 1993) and was confirmed by methylation analysis.

The results of methylation analysis (Table 1) of HEPF1 showed that the galactosyl residues are mainly (1 → 6)-linked with a number of 2,6-*O*-substituted Gal*p* units. The fucosyl residues are completely distributed at non-reducing terminals. The glucosyl residues are mainly (1 → 6)-linked and a small number of terminal residues. The 3-*O*-methylrhamnose is not glycosylated.

The ^1H NMR spectrum (Fig. 3) of the polysaccharide mainly contained signals for four anomeric protons at δ 4.53–5.24, one CH_3–C group (H-6 of Fuc) at δ 1.26 ($J_{5,6}$ 5.4 Hz). Other sugar protons were in the region of δ 3.37–4.25, a signal for an *O*-methyl group at δ 3.50, validated by HMQC spectra.

The ^{13}C NMR spectrum (Fig. 4) of the polysaccharide mainly contained signals for four anomeric carbons at δ

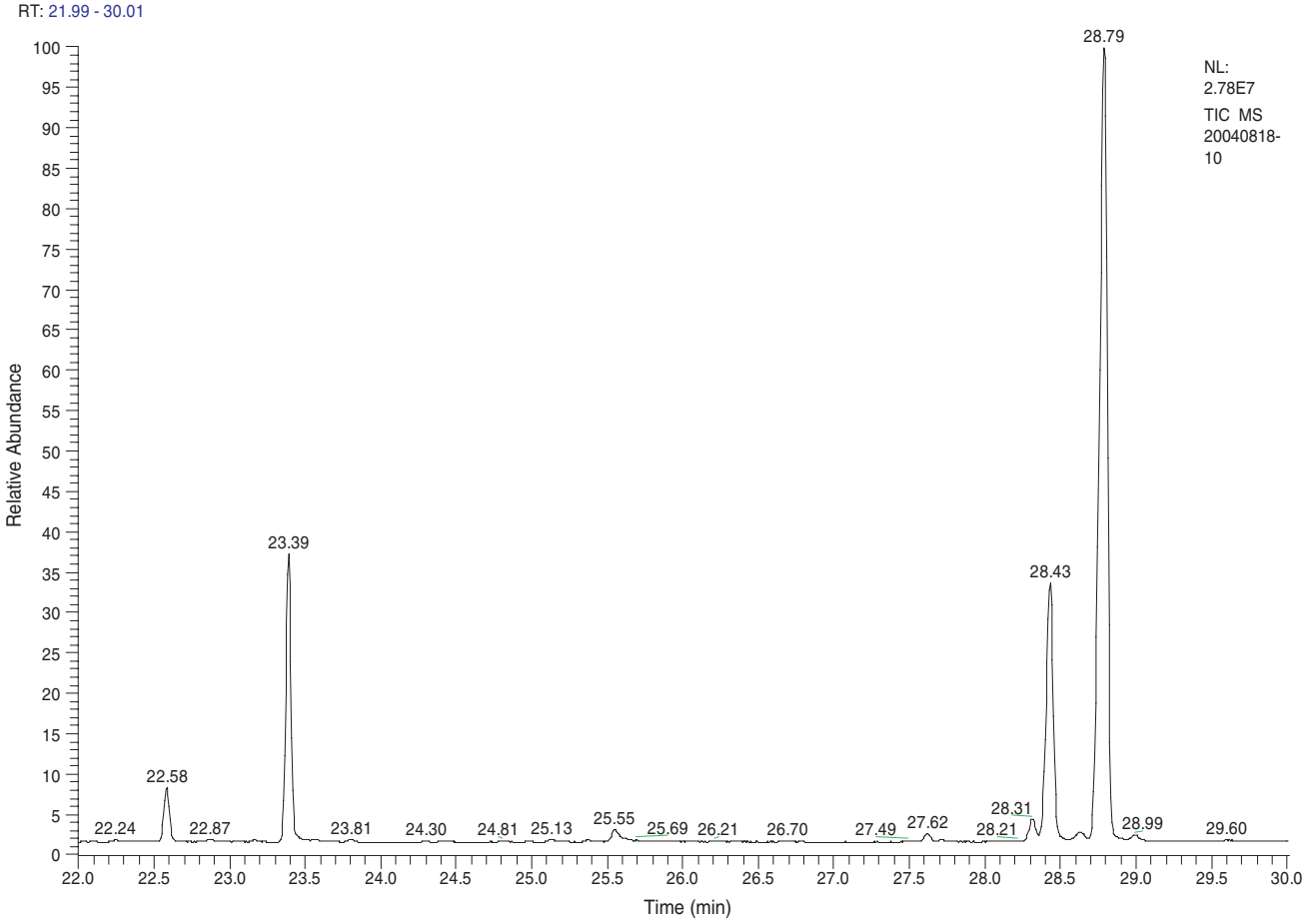

Fig. 1. Total ion chromatogram of HEPF1.

100.25–105.7, one CH_3–C group (C-6 of Fuc) at δ 18.4, and sugar ring carbons linked to oxygen in the region of δ 63.12–77.97. In addition, a minor signal at δ 57.8 could be assigned to an *O*-methyl group and another signal at δ 64.15 was assigned to the C-6 of terminal residue of glucose.

The ^{1}H resonances for H-1, -2 and -3 of residue **a** were assigned from ^{1}H–^{1}H COSY crosspeaks. The assignment of H-4 relied on the TOCSY spectrum. H-5 and H-6 were assigned from the ^{1}H–^{1}H COSY spectrum. The crosspeaks of H-3 and H-6 in the NOESY spectrum, H-6 and C-3 in HMBC spectrum, unambiguously showed that H-5 and H-6 are located on residue **a**. On the basis of the proton assignments, the chemical shifts of C-1–C-6 were readily obtained from the HMQC spectrum. Both the carbon and proton chemical shifts are typical of 6-deoxyhexopyranose, since L-Fuc was the only such sugar identified by GC–MS analysis. H-1 appears as a singlet ($J_{\text{H-1, H-2}} < 3$ Hz) in the ^{1}H NMR spectrum, and H-1/H-2 intra-residue correlations in the NOESY spectrum. Both values indicated an α-configuration at the anomeric center. Thus, residue **a** was identified as α-L-Fuc*p*.

^{1}H resonances for H-1–H-4 of residue **b** were assigned from the ^{1}H–^{1}H COSY and TOCSY spectra. H-5, H-6a and H-6b were assigned from the TOCSY spectrum. In the HMBC spectrum, the crosspeaks of H-1 and C-3, C-5 show that H-5 and H-6 are located on residue **b**. The corresponding ^{13}C resonances were assigned from HMQC spectrum. 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 is evident from the singlet of H-1 as well as the characteristic $J_{\text{H-1, H-2}} < 3$ Hz, H-1/H-2 intra-residue correlations in the NOESY spectrum and the crosspeaks of H-1 and C-3, C-5 in the HMBC spectrum (Stroop, Xu, Retzlaff, Abeygunawardana, & Bush, 2001). The combination of these data identified residue **b** as →2,6)-α-D-Gal*p*.

For residue **c** the ^{1}H resonances for H-1, -2, -3 and -4 were assigned from the crosspeaks in the ^{1}H–^{1}H COSY and TOCSY spectra. The H-5 resonance was assigned from the H-3/H-4 and H-4/H-5 crosspeaks in the NOESY spectrum (Reddy et al., 1998). The H-5, H-6a and H-6b resonances were then obtained from the TOCSY spectrum. ^{13}C resonances were assigned from the HMQC spectrum. H-4 displays strong NOEs to both H-3 and H-5, which indicated that residue **c** is a Gal-type residue. H-1 appears as a singlet ($J_{\text{H-1, H-2}} < 3$ Hz) in the ^{1}H NMR spectrum and H-1/H-2 intra-residue correlations in the NOESY spectrum indicated that residue **c** has an α-configuration. Thus, residue **c** was identified as → 6)-α-D-Gal*p*.

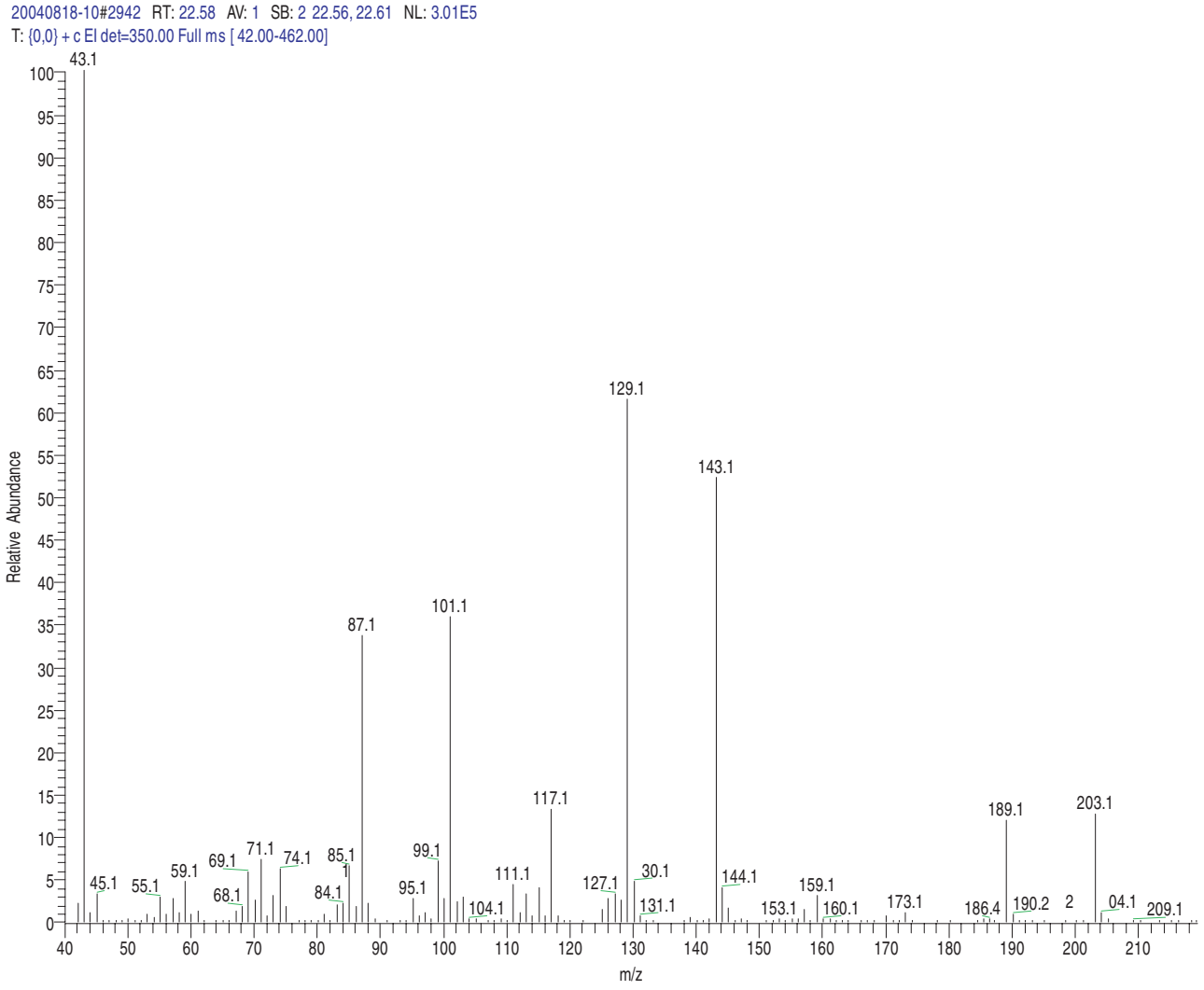

Fig. 2. Mass spectra of 3-*O*-methyl methylrhamnose from HEPF1.

Table 1
Results of the methylation analysis of HEPF1 by GC–MS

Methylated sugar	Substituted sugar unit	Molar ratios	Major mass fragment (*m*/*z*)
2,3,4-Me_3-Rha*p*	Reducing end 3-*O*-Me-Rha*p* unit	0.19	43, 71, 89, 101, 117, 131, 145, 161
2,3,4-Me_3-Fuc*p*	Reducing end Fuc*p* unit	1.00	43, 72, 89, 101, 115, 117, 131, 161, 175
2,3,4,6-Me_4-Glc*p*	Reducing end Glc*p* unit	0.27	43, 71, 87, 101, 117, 129, 145, 161, 205
2,3,4-Me_3-Glc*p*	6-*O*-Substituted Glc*p* unit	1.17	43, 71, 87, 101, 117, 129, 161, 173, 189, 233
2,4-Me_2-Glc*p*	3,6-di-*O*-Substituted Glc*p* unit	0.36	57, 87, 99, 117, 129, 159, 173, 233, 305
2,3,4-Me_3-Gal*p*	6-*O*-Substituted Gal*p* unit	3.11	43, 71, 87, 101, 117, 129, 161, 173, 189, 233
3,4-Me_2-Gal*p*	2,6-di-*O*-Substituted Gal*p* unit	1.50	43, 71, 87, 99, 129, 159, 173, 189, 233, 305

All the ^{1}H resonances for residue **d** were readily assigned from the ^{1}H–^{1}H COSY spectrum and confirmed from the TOCSY spectrum. Magnetisation relayed well through the spin system, as expected for the gluco-configuration, and all crosspeaks were clearly visible (Kondakova et al., 2003).^{13}C resonances were assigned from the HMQC spectrum. H-1 appears as a doublet ($J_{H\text{-}1,\ H\text{-}2}$ 7.34 Hz) in the ^{1}H NMR spectrum and its chemical shifts are less than 4.8 ppm showing that residue **c** has a β-configuration. Thus, residue **d** was identified as →6)-β-D-Glc*p*.

Comparison of the chemical shift data (Table 2) for residues **a**–**d** with those reported for glycosides (Agrawal, 1992) permitted identification of residue **a** as reducing end α-L-Fucp unit, residue **b** as 2,6-di-*O*-substituted α-D-Galp, residue **c** as 6-linked α-D-Galp and residue **d** as →6)-β-D-Glc*p*.

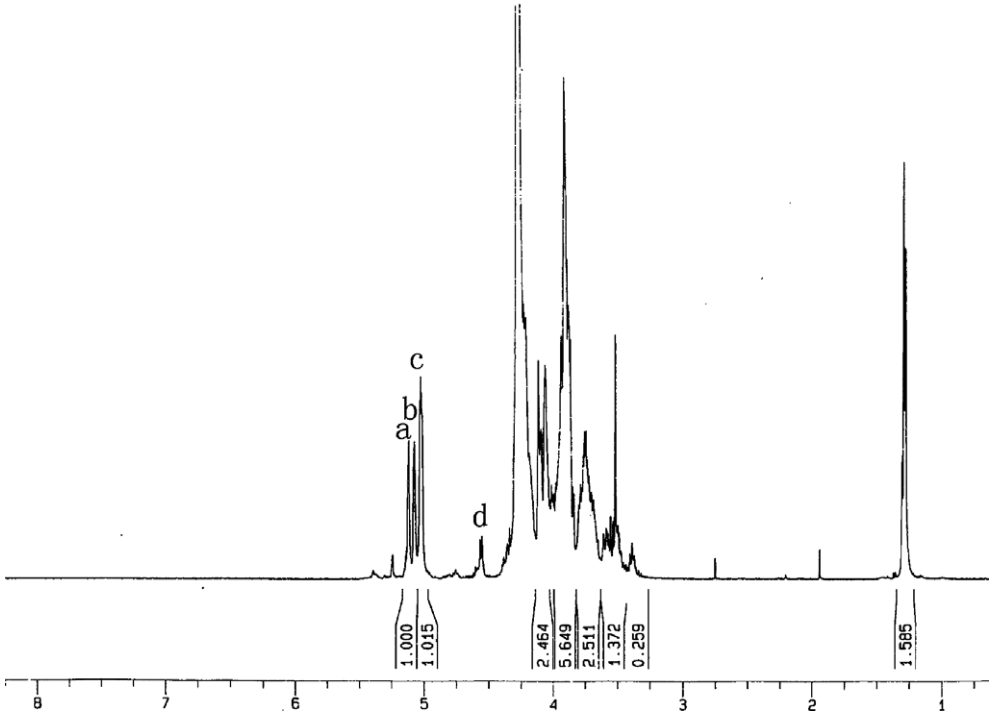

Fig. 3. 500-MHz ^{1}H NMR spectrum of the HEPF1 polysaccharide isolated from *Hericium erinaceus* in D_2O at 60 °C. The anomeric protons are labeled a, b, c, d.

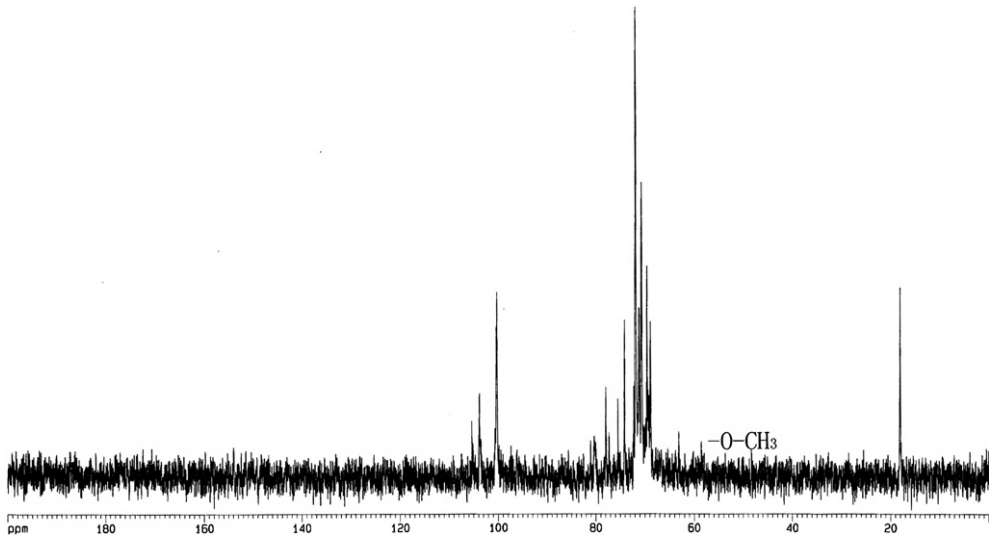

Fig. 4. 500-MHz ^{13}C NMR spectrum of the HEPF1 polysaccharide isolated from *Hericium erinaceus* in D_2O at 60 °C.

The sequence of the residues in the repeating unit was established from the HMBC spectrum, which 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**, between H-1 of residue **c** and C-6 of residue **c**, and between H-1 of residue **c** and C-6 of residue **b**. However, residue **d** does not have cross peaks with other residue.

Taken together, it was proposed that HEPF1 consists of an α-(1 → 6)-D-galactopyranan backbone with a fucosyl units on O-2 of the 2,6-di-*O*-substituted-D-galactosyl units. Moreover, it also contains 6-*O*-substituted-oligo-*β*-D-glucosyl units and a minor terminal 3-*O*-methyl-L-rhamnosyl residues.

In most fungi examined, polysaccharides composed of (1 → 3)-β-D-and (1 → 6)-β-D-glucans, and (1 → 6)-α-D-mannans, have been reported to be the major components of the cell wall and the intercellular matrix, with the latter found mainly in yeast cell walls (Vingradov, Petersen, & Bock, 1998) In contrast, 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). The

Table 2
Chemical shifts data for HEPF1

Residue		Proton or carbon						
		1	2	3	4	5	6a	6b
α-L-Fuc*p* (**a**)	H	5.10	3.84	3.90	3.81	4.18	1.27	
	C	104.2	71.3	71.2	71.5	72.3	18.4	
→2,6)-α-D-Gal*p* (**b**)	H	5.06	3.86	4.09	4.20	4.16	3.69	4.00
	C	100.7	80.5	71.3	69.9	72.4	70.05	
→6)-α-D-Gal*p* (**c**)	H	5.01	3.88	4.05	3.90	4.22	3.73	3.96
	C	100.6	74.6	72.4	72.3	72.1	69.4	
→6)-β-D-Glc*p* (**d**)	H	4.54	3.38	3.52	3.68	3.54	3.71	3.89
	C	105.7	75.5	78.5	76.9	78.5	69.7	

biological effects of these polysaccharides have been widely studied for their immunostimulating and antitumor activities (Tokunaka et al., 2000). However, structures containing an (1 → 6)-linked α-D-galactan backbone and branches composed of fucose and glucose have not been previously reported, HEPF1 is therefore a novel fungal polysaccharide.

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