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Purification and structural investigation of a water-soluble polysaccharide from *Flammulina velutipes*

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ABSTRACT

A novel water-soluble heteropolysaccharide FVP60-B was extracted from the fruiting bodies of *Flammulina velutipes* with boiling water and purified by Sephacryl S-300 and S-400, which molecular weight was estimated to be 1.3×10^4 Da by HPLC. It is composed of L-fucose, D-mannose, D-glucose and D-galactose in a ratio of 1.16:0.82:1.00:3.08. Sugar analysis, methylation analysis together with ^{1}H, ^{13}C and 2D NMR spectroscopy disclosed that FVP60-B is consisted of a α-(1 → 6)-D-galactopyranan backbone with a terminal fucosyl, terminal glucosyl and α-(1 → 6)-D-mannopyranan units on *O*-2 of 2,6-*O*-substituted-D-galactosyl units.

1. Instruction

Flammulina velutipes is a commercially important edible mushroom cultivated widely in many countries, particularly in China and Japan. It has attracted considerable attention in the fields of biochemistry and pharmacology due to its biological activities (Fu, Shieh, & Ho, 2002; Wasser, 2002). It is reported that both the fruiting bodies and the fungal mycelia have bioactive polysaccharides of *F. velutipes* have been proved to be beneficial in immunomodulatory, anti-tumor, and biological activity on hepatocytes, antioxidant activity (Leung, Fung, & Choy, 1997; Ohkuma, Tanaka, & Ikekawa, 1983; Pang et al., 2007). In order to identify correlations between structure and functionality, we have conducted structural studies on polysaccharides from *F. velutipes*. In this paper, the structural elucidation of FVP60-B, a novel neutral polysaccharide purified from the fruiting body of *F. velutipes*, is described by methylation analyses and ^{1}H and ^{13}C NMR spectroscopic analyses, 2D NMR including COSY, TOCSY, HSQC, HMBC and NOESY experiments.

2. Experimental

2.1. Materials

Fruiting bodies were purchased from Jiangshan of Zhejiang Province, PR China. DEAE Sepharose Fast Flow and High-Resolution Sephacryl S-300 and S-400 were purchased from GE Healthcare. Dextrans, trifluoroacetic acids (TFA) and monosaccharide standards (D-galactose, D-arabinose, L-fucose, L-rhamnose, D-manonose, D-xylose, D-glucose) were from Sigma. All the other reagents were of A.R. grade and made in China. HPLC was carried out on a waters 1525 HPLC system (1525 HPLC pump, 2414 refractive index detector). GC was carried out using an Agilent 7890N instrument. 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 dried fruiting bodies of *F. velutipes* were cut into small pieces and extracted with boiling water thrice (2 h for each). The supernatant was combined and concentrated into one-tenth of the original volume, and 95% EtOH was added slowly to final alcohol concentration reached 40%. The precipitated was obtained by centrifugation (10,000 rpm, 10 min, 4 °C), and 95% EtOH was continually added slowly to final concentration of 60%. The resulting precipitate was collected, dissolved in distilled water and lyophilized, and defined as FVP60. A portion of FVP60 was dissolved in water and insoluble residue was removed by centrifugation. The supernatant was injected to a DEAE Sepharose Fast Flow column (XK 26 mm × 100 cm), eluted first with water, and then followed stepwise by 0.1–2 M gradient of NaCl, respectively. The fractions were collected by a fraction collector and compounds were detected by means of the phenol-sulfuric acid assay (Zhang, 1999), FVP60-B was obtained by elution with water, and then further purified using High-Resolution Sephacryl S-300 and S-400 (XK 26 mm × 100 cm) gel-permeation chromatography. The main fraction was collected, concentrated and lyophilized to get a white

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purified *F. velutipes* polysaccharide (FVP60-B), whose molecular weight range was detected firstly on a column SN of TSK-gel PWXL G4000.

2.3. Determination of purity and molecular weight

The homogeneity and molecular weight of FVP60-B were determined by high performance liquid chromatography (HPLC) on an Agilent 1525 system equipped with a TSK-gel PWXL G4000 column and a refractive index detector (RID). 10 μL of sample solution (2.0 mg/mL) was injected in each run, with distilled water as the mobile phase at a flow rate of 1. 0 mL/min. The column was kept at 30.0 ± 0.1 °C. The linear regression was calibrated with T-series Dextrans standards (M_w 1000, 5000, 12,000, 80 k, 150 k, 270 k and 670 kDa).

2.4. Spectroscopic methods

The ultraviolet spectrum of FVP60-B was recorded with a UV-2450 UV–visible spectrophotometer (Shimadzu Co., Kyoto, Japan). The Fourier-transform infrared spectrum of FVP60-B was measured on a Nicolet 6700 FT-IR spectrometer (Madison, WI, USA) using the KBr disk method.

2.5. Sugar analysis

The identification and quantification of the monosaccharide of FVP60-B was achieved by GC analysis. FVP60-B (2 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA, 4 mL) at 110 °C for 2 h. Then the hydrolyzed products were reduced with $NaBH_4$ (20 mg) and acetylated with acetic anhydride (Albersheim, Nevins, English, & Karr, 1967). The resulting alditol acetates were analyzed by gas chromatography (GC) using an Agilent 7890N instrument equipped with an HP-5 capillary column (30 m × 0.32 mm × 0.25 μm) and a flame-ionization detector (FID) at a temperature program as follows: the oven temperature was initially set at 120 °C, increasing to 240 °C at a rate of 10 °C/min and then held at 240 °C for 6.5 min. The heater temperatures of the injector and detector were both at 250 °C. Nitrogen was used as the carrier gas. Quantitation was carried out from the peak area, using response factors of instrument.

2.6. Methylation analysis

FVP60-B (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 spectrum. The permethylated polysaccharide was hydrolyzed by treatment with HCO_2H (88%, 3 mL) at 100 °C for 3 h, evaporated to dryness and further hydrolyzed with 2 M TFA (4 mL) at 100 °C for 6 h. The partially methylated sugar in the hydrolysate were reacted with $NaBH_4$ and acetylated with AC_2O, and the resulting mixture of methylated alditol acetates were analyzed by GC–MS.

2.7. NMR analysis

FVP60-B (10 mg) was dried in a vacuum over P_2O_5 for 72 h, and then exchanged with deuterium by lyophilizing with D_2O (0.5 mL) for three times. ^1H NMR (25 °C, 60 °C) and ^{13}C NMR (60 °C) spectra were determined in 5-mm tubes using a Varian INOVA 500 NMR spectrometer (TOSO, Tokyo, Japan). ^1H Chemical shifts were referenced to residual HDO at δ 4.78 ppm (25 °C) as the 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), heteronuclear single quantum correlation spectroscopy (HSQC) 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

3.1. Isolation, purification, and composition of FVP60-B

The water-soluble polysaccharide termed FVP60-B was purified by High Resolution Sephacryl S-300 and S-400 gel-permeation chromatography. High-performance liquid chromatography (HPLC) of FVP60-B produced a single symmetrical peak, indicating it was a homogeneous polysaccharide (Fig. 1). Correlation with the calibration curve of Dextran standards indicated that its molecular weight was about 1.3×10^4 Da. Lack of absorption at 280 nm by UV scanning indicated that FVP60-B contained no protein. The infrared spectrum of FVP60-B displayed a broad stretching intense characteristic peak at around 3428.4 cm^{-1} for the hydroxyl group of the polysaccharide, and a C–H stretching band at 2923 cm^{-1}. The relatively absorption peak at 1634 cm^{-1} and the weak one at 1382 cm^{-1} also indicated the characteristic IR absorption of polysaccharides (Ge, Zhang, & Sun, 2009). No absorption peaks at 1730 cm^{-1} indicated that there were no uronic acids. Sugar compositional analysis of FVP60-B determined by GC indicated that it consists of L-fucose, D-mannose, D-glucose and D-galactose in a ratio of 1.16:0.82:1.00:3.08.

3.2. Structural characterization of FVP60-B

The inter-glycosidic linkages between monosaccharide residues of FVP60-B were investigated by methylation analysis. The polysaccharide was methylated twice and after acid hydrolysis, methylated sugars were converted to partially methylated alditol acetates. Total ion chromatogram (Fig. 2) showed that mainly six peaks. Major mass fragment showed that it isn't sugar residue at 5.80 min. Other peaks all possess major mass fragment of methylated sugar.

The results of methylation analysis (Table 1) of FVP60-B 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 terminal residues.

The ^1H NMR spectrum (Fig. 3) of the polysaccharide mainly contained signals for five anomeric protons at δ 5.00–5.17, one CH_3–C group (H-6 of Fuc) at δ 1.28 ($J_{5,6}$ = 5.0 Hz). Other sugar protons were in the region of δ 3.30–4.20. The ^{13}C NMR spectrum (Fig. 4) of the polysaccharide mainly contained signal for anomeric carbons at δ 5.03–5.17, one CH_3–C group (C-6 of Fuc) at δ 18.34, and sugar ring carbons linked to oxygen in the region of δ 63.89–80.50.

^1H resonances for H-1, H-2, H-3 and H-4 residue **a** were assigned from the cross-peaks in the ^1H–^1H COSY and TOCSY spectra. H-5, H-6a and H-6b were assigned from the ^1H–^1H COSY, TOCSY and HMBC spectra. The carbon chemical shifts from the C-1 to C-6 were assigned from the HSQC spectrum (Table 2). The manno configuration for residue **a** was supported from a relatively small coupling constant value of $J_{1,2}$ < 3 Hz and a large coupling constant value of $J_{4,5}$ = 9.5 Hz. A small $J_{1,2}$ values for the D-mannosyl residue did not give information about the anomeric configuration (Paramonov et al., 2001). A NOESY experiment revealed

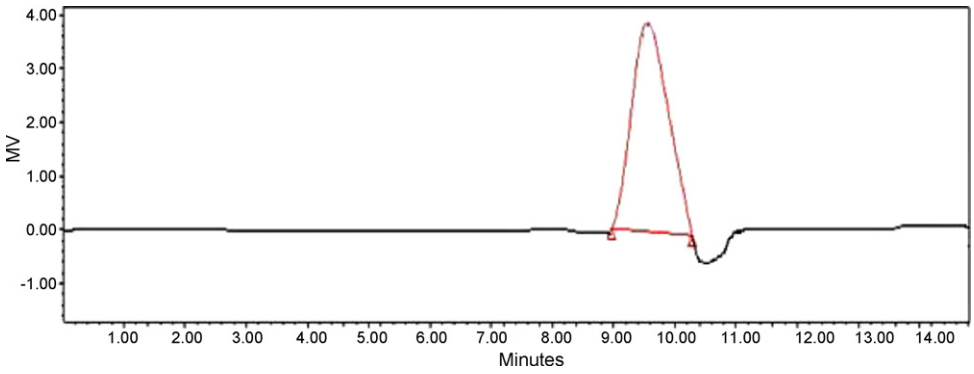

Fig. 1. HPLC of FVP60-B on TSK-gel PWXL G4000 column.

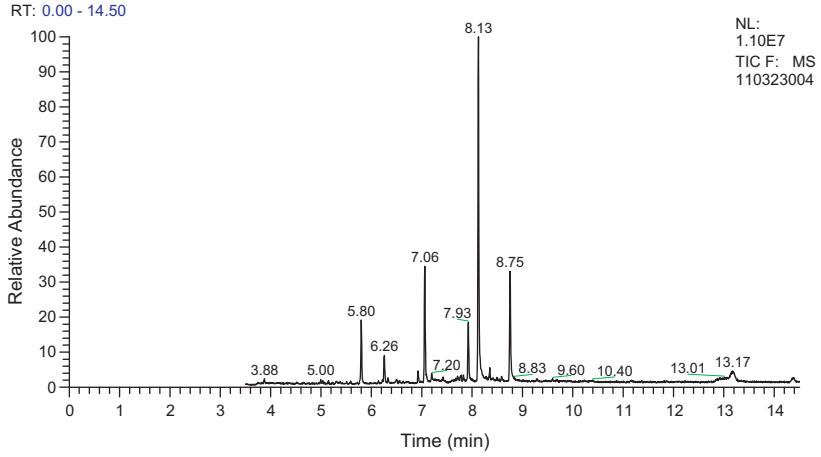

Fig. 2. Total ion chromatogram of FVP60-B by GC–MS.

Table 1
The methylation analysis of FVP60-B isolated from the fruiting bodies of *F. velutipes*.

Retent time	Methylated sugar	Substituted sugar unit	Molar ratios	Major mass fragment (*m*/*z*)
6.26	2,3,4-Me_3-Fuc*p*	Reducing end Fuc*p* unit	0.24	43, 72, 89, 101, 115, 117, 131, 161, 175
7.06	2,3,4,6-Me_4-Glc*p*	Reducing end Glc*p* unit	1.00	43, 71, 87, 101, 117, 129, 145, 161, 205
7.93	2,3,4-Me_3-Man*p*	6-*O*-Substituted Man*p* unit	0.48	43, 71, 87, 101, 117, 129, 161, 173, 189, 233
8.13	2,3,4-Me_3-Gal*p*	6-*O*-Substituted Gal*p* unit	3.10	43, 71, 87, 101, 117, 129, 161, 173, 189, 233
8.75	3,4-Me_2-Gal*p*	2,6-di-*O*-Substituted Gal*p* unit	1.11	43, 71, 87, 99, 129, 159, 173, 189, 233, 305

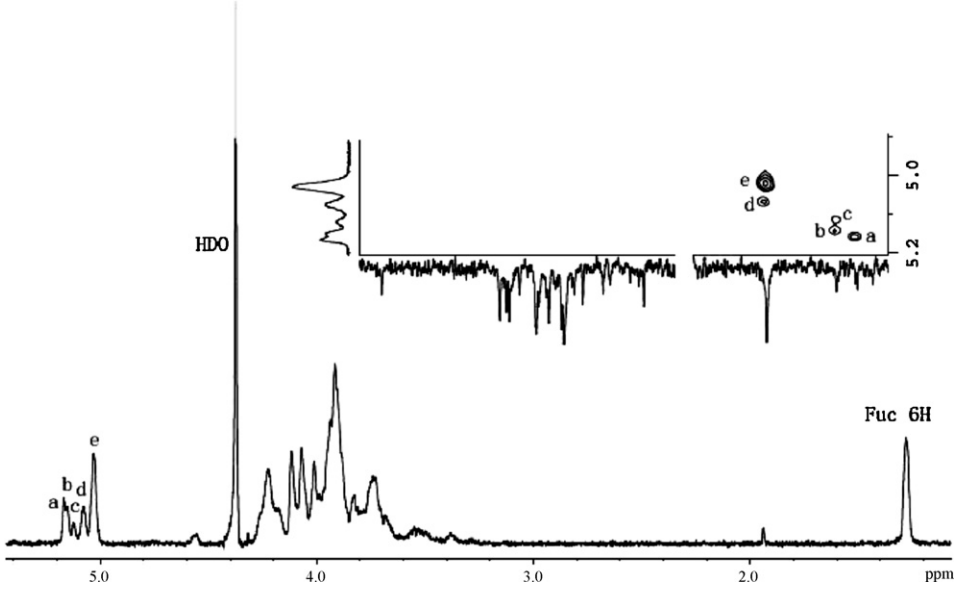

Fig. 3. 500-MHz ^{1}H NMR spectrum of FVP60-B in D_2O at 60 °C verified by partial of HSQC. The anomeric protons are labeled (a)–(e).

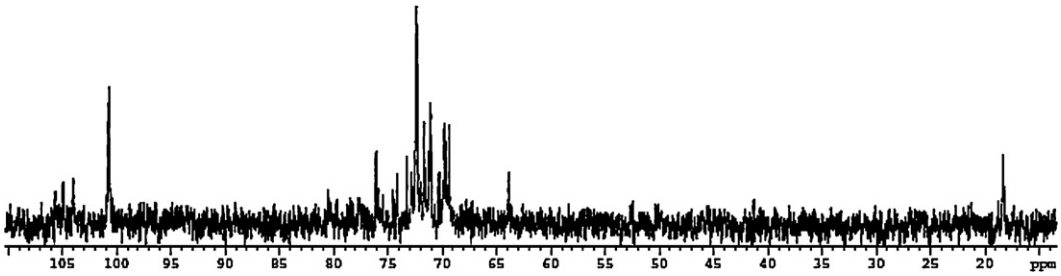

Fig. 4. 500-MHz ^{13}C NMR spectrum of FVP60-B in D_2O at 60 °C.

Table 2
Chemical shifts data for FVP60-B isolated from the fruiting bodies of *F. velutipes*.

Residue		Proton or carbon						
		1	2	3	4	5	6a	6b
→6)-α-D-Man*p* (**a**)	H	5.17	4.12	3.95	3.83	3.74	3.97	4.21
	C	104.95	72.65	69.83	76.00	69.39	69.90	
α-D-Glc*p* (**b**)	H	5.16	3.98	3.95	4.10	4.01	3.80	3.93
	C	103.98	72.20	73.25	71.21	74.20	63.89	
α-L-Fuc*p* (**c**)	H	5.12	3.87	3.92	3.85	4.21	1.28	
	C	103.98	71.15	72.30	71.21	69.80	18.34	
→2,6)-α-D-Gal*p*(**d**)	H	5.08	3.88	4.10	4.21	4.18	3.70	4.01
	C	100.70	80.50	71.21	69.80	71.90	69.99	
→6)-α-D-Gal*p*(**e**)	H	5.03	3.88	4.07	3.91	4.23	3.74	3.97
	C	100.70	74.45	72.40	72.30	71.55	69.39	

inter-residue correlations between H-1 and H-2, indicating that residue **a** was α-configuration (Senchenkova et al., 2003). The downfield shifts of the C-6 (δ 69.90) carbon signals with respect to standard values for glycopyrannoses indicate that residue **a** was →6)-α-D-Man*p*-(1→.

Chemical shifts of residue **b** were assigned according to ^1H–^1H COSY and TOCSY spectra. Large coupling constants was $J_{H\text{-}2,\,H\text{-}3}$ (8–10 Hz) and $J_{H\text{-}3,\,H\text{-}4}$ (8–10 Hz) indicated that residue **b** was a gluco-configuration. ^{13}C resonances were assigned from the HSQC spectrum (Table 2). H-1 appears as a singlet ($J_{1,2}$ < 3 Hz) in the ^1H NMR spectrum and H-1/H-2 intra residue correlations in the NOESY spectrum showing that residue **b** has an α-configuration. The combination of these data identified residue **b** as α-D-Glc*p*.

The ^1H resonances for H-1, 2, 3 of residue **c** were assigned from ^1H–^1H COSY spectrum, and the assignment of H-4 was based on the TOCSY spectrum. H-5 and H-6 were assigned from ^1H–^1H COSY spectrum. Both H-3 and H-4 correlated with a signal δ 4.21, and H-4 gave a <!– no-mfc –>NOE<!– /no-mfc –> signal at δ 1.28 were located on residue **c**. On basis of the proton assignments, the chemical shifts of C-1 to C-6 were readily obtained from the HSQC spectrum (Table 2). Both the carbon and 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 ($J_{1,2}$ < 3 Hz) in the ^1H 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 **c** was identified as α-L-Fuc*p*.

^1H resonances for H-1 to H-4 of residue **d** were assigned from the ^1H–^1H COSY and TOCSY spectra. H-5 and H-6 were assigned from the TOCSY spectrum. The crosspeaks of H-1 and C-3, C-5 of the HMBC spectrum showed that H-5 and H-6 are located on residue **d**. The chemical shifts of C-1 to C-6 were readily obtained from the HSQC spectrum (Table 2). The H-4/5 coupling constant was small in the ^1H–^1H COSY spectrum, as expected for a Gal-type residue (Staaf, Urbina, Weintraub, & Widmalml, 1999). H-1 appears as a singlet ($J_{1,2}$ < 3 Hz) in the ^1H NMR spectrum and H-1/H-2 intra residue correlations in the NOESY spectrum indicted that residue d has an α-configuration. The downfield shifts of the C-2 (δ 80.50) and C-6 (δ 69.99) carbon signals with respect to standard values for glycopyrannoses indicate that residue **d** was →2,6)-α-D-Gal*p*-(1→.

For residue **e** the ^1H resonances for H-1, H-2, H-3 and H-4 were assigned from the crosspeaks in the ^1H–^1H 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-6 resonances were then obtained from the TOCSY spectrum. The corresponding ^{13}C resonances were assigned from the HSQC spectrum (Table 2). H-4 displays strong NOEs to both H-3 and H-5, which indicated that residue **e** is a Gal-type residue. Residue **e** had an α-configuration at its anomeric center, which is evident from the 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 downfield shifts of the C-6 (δ 69.39) carbon signals with respect to standard values for glycopyrannoses indicate that residue **e** was →6)-α-D-Gal*p*-(1→.

The sequence of glycosyl residues was determined from NOESY studies followed by confirmation with HMBC experiments. Inter-residue <!– no-mfc –>NOE<!– /no-mfc –> correlations (Table 3)

Table 3
Interglycosidic correlations from NOESY spectra of FVP60-B isolated from the fruiting bodies of *F. velutipes*.

Residue	Proton	Intra-correlation
→6)-α-D-Man*p* (**a**)	H-1	**4.12** (**a**; H-2), **4.21** (**a**; **H6b**)
α-D-Glc*p* (**b**)	H-1	3.98 (**b**; H-2), **3.88** (**d**; **H-2**)
α-L-Fuc*p* (**c**)	H-1	3.87 (**c**; H-2), **3.88** (d; **H-2**)
→2,6)-α-D-Gal*p*(**d**)	H-1	3.88 (**d**; H-2), 4.10 (**d**; H-3), 4.21 (**d**; H-4), **3.70** (**d**; **H-6a**), **4.01** (**d**; **H-6b**)
	H-4	4.10 (**d**; H-3), 4.18 (**d**; H-5)
→6)-α-D-Gal*p*(**e**)	H-1	3.88 (**e**; H-2), 4.23 (**e**; H-5), **3.74** (**e**; **H-6a**), **4.01**(**d**; **H-6b**)
	H-4	4.07 (**e**; H-3), 4.23 (**e**; H-5)

[a] Inter-residue NOEs are shown in bold font.

Table 4
Two-and three-bond $^{1}H-^{13}C$ correlations for the FVP60-B isolated from the fruiting bodies of *F. velutipes*.

Residue	Proton	Proton correlation
→6)-α-D-Manp (a)	H-1	73.25 (**a**; C-2), 76.09 (a; C-4), 69.90 (a; C-6), 80.50(d; C-2)
α-D-Glc*p* (**b**)	H-1	69.83 (b; C-3), 80.50(d; C-2)
α-L-Fuc*p* (**c**)	H-1	72.30 (c; C-3), 69.80 (b; C-5), 80.50(d; C-2)
→2,6)-α-D-Gal*p*(**d**)	H-1	71.21 (d; C-3), 71.90 (d; C-5), 69.99(d; C-6)
→6)-α-D-Gal*p*(**e**)	H-1	72.40 (e; C-3), 71.55 (e; C-5), 63.89(e; C-6)

were observed between H-1 of residue **a** and H-6 of residue **a**, between H-1 of residue **b** and H-2 of residue **d**, between H-1 of residue **c** and H-2 of residue **d**, between H-1 of residue **d** and H-6 of residue **d**, between H-1 of residue **e** and H-6a of residue **d** and H-6b of residue **e**. HMBC experiments (Table 4) showed clear correlations between H-1 of residue **a** and C-6 of residue **a** and C-2 of residue **d**, between H-1 of residue **b** and C-2 of residue **d,** between H-1 of residue **c** and C-2 of residue **d**, between H-1 of residue **d** and C-6 of residue **d,** between H-1 of residue **e** and C-6 of residue **e**.

Based on the data presented above, it demonstrated that FVB60-B consisted of a α-(1 → 6)-D-galactopyranan backbone with a terminal fucosyl, terminal glucosyl and α-(1 → 6)-D-mannopyranan units on *O*-2 of 2,6-*O*-substituted-D-galactosyl units.

4. Conclusion

According to the results above, FVP60-B was isolated from the fruiting bodies of *F. velutipes* with boiling water extraction and purified by DEAE Sepharose Fast Flow column and High-Resolution Sephacryl S-300, 400. Its molecular weight was measured to be 1.3×10^4 Da by using HPLC. Gas chromatography analysis suggested that FVP60-B is composed of L-fucose, D-mannose, D-glucose and D-galactose in a ratio of 1.16:0.82:1.00:3.08. Sugar analysis, methylation analysis together with ^{1}H and ^{13}C NMR spectroscopy and 2D NMR established that FVP60-B is consisted of a α-(1 → 6)-D-galactopyranan backbone with a terminal fucosyl, terminal glucosyl and α-(1 → 6)-D-mannopyranan units on *O*-2 of 2,6-*O*-substituted-D-galactosyl units.

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