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Purification, characterization and biological activity on hepatocytes of a polysaccharide from *Flammulina velutipes* mycelium

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Abstract

A water-soluble polysaccharide named as FVP2 has been isolated from *Flammulina velutipes* mycelium by hot water extraction, anion-exchange and gel-permeation chromatography. Structure study shows FVP2 is an α-(1→4)-D-glucan, with a single α-D-glucan at the C-6 position approximately every seven residues, along the main chain. The weight-average molecular weight is 1.89×10^4 Da. Its biological activity was tested on hepatocytes. *In vitro* study indicates FVP2 can enhance the livability of primary culture of mouse hepatocytes and decrease the release of ALT as well as apoptosis of hepatocytes after CCl_4 intoxication.

Keywords: *Flammulina velutipes*; Polysaccharide; Mycelium; Structure; D-Glucan; Hepatocyte

1. Introduction

Malignant neoplasm is presently one of the diseases threatening people's health. Recently, several Chinese herbal medicines, which possess anti-tumor and/or immune-stimulating properties, have been obtained increasing attention and used clinically in prevention and treatment of cancer as adjuvants such as lingzhi (*Ganoderma lucidum*), shiitake (*Lentinus edodes*), yiner (*Tremella fuciformis*), *Coriolus versicolor*, and krestin (*Cordyceps sinensis*) (Cui & Chisti, 2003; Wu, Sun, & Pan, 2006; Zhang, Cui, Cheung, & Wang, 2007). Many species in polysaccharide, a big class of natural macromolecules, have been found to carry significant biological activities. It has been reported that polysaccharides derived from medicinal mushrooms have activities of anti-tumor, anti-infection, anti-aging, anti-radiation etc. All these activities are due to its contribution to enhance immune function of the human body (Cui & Chisti, 2003; Leung, Liu, Koon, & Fung, 2006; Zhang et al., 2007).

Flammulina velutipes, ascribed to Eumyeota, Basidiomycotina, Hgmenomycetes, Holobaeidiomycetidae, Agaricel, Tricholomataceae, *Flammulina*, is one of the most popular edible fugus in China and Japan. Its production and consumption ranks fourth in the edible mushrooms (Leifa, Pandey, & Soccol, 2001). The first study of *F. velutipes* polysaccharide (FVP) was reported by Kamasuka et al. (Kamasuka, Momoki, & Sakai, 1968). Since then several other polysaccharides have been isolated from *F. velutipes* fruit bodies and mycelium (Ikekawa et al., 1982; Leung, Fung, & Chay, 1997; Ohkuma, Tanaka, & Ikekawa, 1983). All these studies reveal FVPs stimulate concanavalin A-induced mitogenic activity of T lymphocytes, promotes T-cell to produce antibodies and also induces expression of interferon, indicating that FVPs possess a great potential. However, there is no report what the exact active compound in FVP is and its function on liver. In the present study, we report a new polysaccharide isolated from *F. velutipes* mycelium and its structure characteristic as well as its biological effects on hepatocytes.

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2. Materials and methods

2.1. Materials

Flammulina velutipes mycelium (Jiangsu Shenhua Pharmaceutical Co. Ltd., China), was air dried and ground. Dextran standards of T-500, T-70, T-40, T-10 were purchased from Pharmacia Co. Ltd. Hepatocyte growth factor (HGF) was purchased from Nanjing, Nanjing University Pharmaceutical Co. Ltd. (China).

2.2. General methods

The specific rotation was determined at 20 ± 1 °C with an automatic polarimeter (Model WZZ-2, China). The FTIR spectra was recorded on a Nicolet Impact 410 spectrophotometer with KBr pellets. Total carbohydrate content was determined by the anthrone–sulphuric acid method as D-glucose equivalents (Scott & Melvin, 1953). Protein was analyzed by the Coomassie Brilliant G-250 method. GC was analyzed on a Hewlett-Packard model 6890 instrument equipped with a capillary column of HP-5.5% phenyl methyl siloxane (30 m × 0.25 mm × 0.25 μm) and a flame-ionization detector, and programmed from 150 to 220 °C at 2 °C/min and from 220 to 280 °C at 30 °C/min. GC–MS was conducted with a UAR1AN Saturn 2000 GC/MS/MS instrument, using a DB5 capillary column (30 m × 0.25 mm × 0.25 μm). The column temperature was held at 140 °C for 3 min, increased to 250 °C at 3 °C/min, and kept for 10 min. The ionization potential was 70 eV and the temperature of the ion source was 220 °C. All NMR (^{1}H, ^{13}C, and HSQC) experiments were accomplished at 30 °C in D_2O on a Bruker AV-500 NMR spectrometer (Liu et al., 2007).

2.3. Isolation and purification of Flammulina velutipes polysaccharides

Flammulina velutipes mycelium (50 g) was extracted with 1000 mL water for 8 h at 70–80 °C. The extraction was collected by centrifugation and concentrated till 200 mL under reduced pressure, then protein was removed with Sevag method (Staub, 1965), followed by dialyzing against distilled water. The nondialyzable phase was diluted with three times of volume with 95% EtOH and kept at 4 °C for 4 h. After centrifugation, the resulting precipitate was washed sequentially with ethanol, acetone, ether, and vacuum-dried. The yield of crude polysaccharide was 9.5% of the dried material.

The crude polysaccharide was applied to a DEAE–Sephadex A-50 column (2.0 × 44 cm), eluted with H_2O. Fractions were collected and measured for carbohydrate by the anthrone–sulphuric acid method (Scott & Melvin, 1953). Further purified on a Sephacryl S-400 column (1.5 × 65 cm) eluted with H_2O at a flow rate of 9 mL/h. Two polysaccharide fractions named as FVP1 and FVP2 were separated. The yield of FVP1 and FVP2 were about 16.6% and 43.7% from the crude polysaccharide, respectively, of which the FVP2 was used in the subsequent studies.

2.4. Homogeneity and molecular weight determination

The molecular weight of FVP2 was determined by gel chromatographic technique (Rodriguez & Vanderwieles, 1979; Wu et al., 2006). Measurements were carried out on a Sephacryl S-400 column (1.0 × 100 cm), eluted with H_2O at a flow rate of 9 mL/h. A series of different weight-average molecular weights of standard dextrans T-500, T-70, T-40, and T-10 were prepared as 0.1% (w/v) solutions and 1 mL of solution was subjected in each run, and then the elution volumes were plotted against the logarithms of their respective molecular weights. A solution of FVP2 (3 mg) in distilled water (0.5 mL) was passed through the column. The elution volume of FVP2 was then plotted in the same graph, and the weight-average molecular weight of FVP2 was obtained. The homogeneity of FVP2 was determined on an Agilent 1100 HPLC system equipped with a Shodex SUGAR KS-805 column (8 × 300 mm) according to the protocol of gel-permeation chromatography (GPC) previously described by Yang et al. (Yang et al., 2005), and was also measured by polyacyrlamide gel electrophoresis.

2.5. Monosaccharide composition

The polysaccharide (10 mg) was hydrolyzed in 2 M trifluoroacetic acid (2 mL) at 100 °C for 8 h. The hydrolysate was co-analyzed by TLC and GC methods. TLC was performed on thin layer plate (silica gel G) with a solvent system of *o*-butanol, acetoacetate, iso-propanol and H_2O in a ratio of 7:4:7:2 (v/v). D-mannose, D-glucose, D-fucose, D-arabinose and D-rhamnose were used as standard sugars. Sugar spots were visualized by spraying *o*-phthalic acid reagent. For GC analysis, the hydrolysate of FVP2 was converted into its respective alditol acetates and analyzed by gas chromatography with myo-inositol as the internal standard (Honda, Suzuki, Kakehi, Honda, & Takai, 1981).

2.6. Methylation analysis

The polysaccharide was methylated three times by the Needs and Selvendran method (Needs & Selvendran, 1993). The methylated product as depolymerized with 90% HCOOH at 100 °C for 6 h and further hydrolyzed with 2 M TFA at 100 °C for 2 h. The partially methylated residues were reduced and acetylated (Blakeney, Harris, Henry, & Stone, 1983). The resulting products were analyzed by GC–MS. The GC temperature program was carried out as described previously (Bao, Liu, Fang, & Li, 2001). Partially methylated alditol acetates were identified by their fragment ions in EI-MS and by relative retention times in GC, and the molar ratios were estimated from

the peak areas and response factors (Sweet, Shapiro, & Albersheim, 1975).

2.7. Periodate oxidation and smith degradation

The polysaccharide (10 mg) was dissolved in 0.015 M sodium metaperiodate (25 mL) and kept at 5 °C in the dark for 7 days. The oxidation was stopped by addition of ethylene glycol (1 mL). Consumption of $NaIO_4$ was measured by a spectrophotometric method and HCOOH production was determined by titration with 0.01 M NaOH. The reaction mixture was dialyzed against distilled water, and the nondialysate was reduced by $NaBH_4$ (30 mg, 18 h), neutralized with 0.1 M acetic acid, dialyzed, and the nondialysate was lyophilized and then hydrolyzed with 2 M TFA at 100 °C for 8 h. The hydrolysate was analyzed by TLC as previously described (Yang et al., 2005), product spots were visualized by spraying with 0.015 M $NaIO_4$ by standing for 8 min and then sprayed with benzidine developer (0.92 g of benzidine dissolved in 50 mL of 50% ethanol, 5 mL of 0.2 M HCl and 10 mL of acetone were added into the solution).

2.8. Partial hydrolysis

FVP2 was partially hydrolyzed with 0.1 M TFA at 100 °C for 18 h. After dialysis of the hydrolysate with a M_r 3 kDa cut-off membrane, a nondialysate portion (FVP2-p) and a dialysate portion were obtained. The periodate oxidation and Smith degradation of FVP2-p was followed the same procedure as mentioned above. The dialysate portion was directly converted into its respective alditol acetates and analyzed by gas chromatography (Honda et al., 1981).

2.9. Assay of biological activity on hepatocytes

2.9.1. Hepatocytes preparation

Male Kunming mice (4- to 5-weeks-old) were purchased from the Experimental Animal Center, China Pharmaceutical University. Animal quality certificate Number is SCXK (HU) 2002-0010. Hepatocytes were isolated from mice by using a modified two-step perfusion method (Wu, Cao, Jiang, Yu, & Xu, 2001). In brief, mice were given 40 mg/kg pentobarbital intraperitoneally. The liver was perfused sequentially with D-Hanks solution and 0.05% Collagenase IV via the portal vein. Then the liver was excised rapidly from the abdominal cavity and dispersed into RPMI-1640 medium. The cell suspension was filtered through a 200-gauze mesh, and parenchymal hepatocytes were separated from nonparenchymal cells by differential centrifugation at 50 g for 2 min. After removing dead cells and debris, the hepatocytes were incubated in RPMI-1640 medium containing 100 U/mL penicillin–streptomycin and 10% fetal bovine serum. The hepatocytes used in experiments were approximately 80% viable as estimated by trypan blue exclusion.

2.9.2. Influence of FVP2 on the hepatocytes

0.2 mL hepatocytes suspended in RPMI-1640 medium were loaded on a 96-well flat-bottomed microculture plate at a density of 1.5×10^5 cells/mL and incubated at 37 °C with 5% CO_2. Two hours later, various concentrations of FVP2 (25, 50, 100, 200 μg/mL), RPMI-1640 medium and 200 μg/mL of HGF as negative and positive control group, were added to the wells, respectively (Noji et al., 1990). After 2 h of culture, cell growth was evaluated with MTT method (Mosmann, 1983).

2.9.3. Protective effect of FVP2 on the hepatocytes damage by carbon tetrachloride (CCl_4)

To determine whether FVP2 could protect hepatocytes against CCl_4 damage, 0.2 mL hepatocytes suspended in RPMI-1640 medium were loaded on a 96-well flat-bottomed microculture plate at a density of 1.5×10^5 cells/mL and cultured in a humidified incubator at 37 °C with 5% CO_2 for 2 h. Then various concentrations of FVP2 (25, 50, 100, 200 μg/mL), RPMI-1640 medium (control group) and 200 μg/mL of HGF (positive group) were added to the wells, respectively. One hour later, CCl_4 was added to the wells at the final concentration of 10 mmol/L. After 2 h of culture, the content of alanine transaminase (ALT) in the culture medium was measured by Reitman–Frankel method.

2.10. Statistical analysis

The data were expressed as means ± SD. Significance of difference was tested by Student's *t*-test, the levels of significance are indicated as difference letters.

3. Results and discussion

3.1. Isolation and structural analysis

The crude polysaccharide extracted from the mycelium of *F. velutipes* was fractionated and sequentially purified through DEAE–Sephadex A-50 and Sephacryl S-400 column eluted with H_2O, leading to the isolation of two fractions FVP1 and FVP2. The main fraction (FVP2) was chosen for subsequent analysis.

FVP2 appeared as a white powder, $[\alpha]_D^{20}$ +160.19° (*c* 0.2, H_2O). A single and symmetric sharp peak appears in gel-permeation chromatography (GPC) profile (Fig. 1). Also a single spot was observed after polyacyrlamide gel electrophoresis. Both indicate that FVP2 is a homogeneous polysaccharide. The weight-average molecular weight of FVP2 was determined by gel-filtration technique using different carbohydrate markers passing through a Sephacryl S-400 column and found to be 1.89×10^4 Da. The total sugar content of FVP2 is 99.6% measured by the anthrone–sulphuric acid method. Reaction with Coomassie Brilliant G-250 was negative and no absorption at 280 or 260 nm in the UV spectrum, indicating that the preparation of FVP2 contained no protein or nucleic acid.

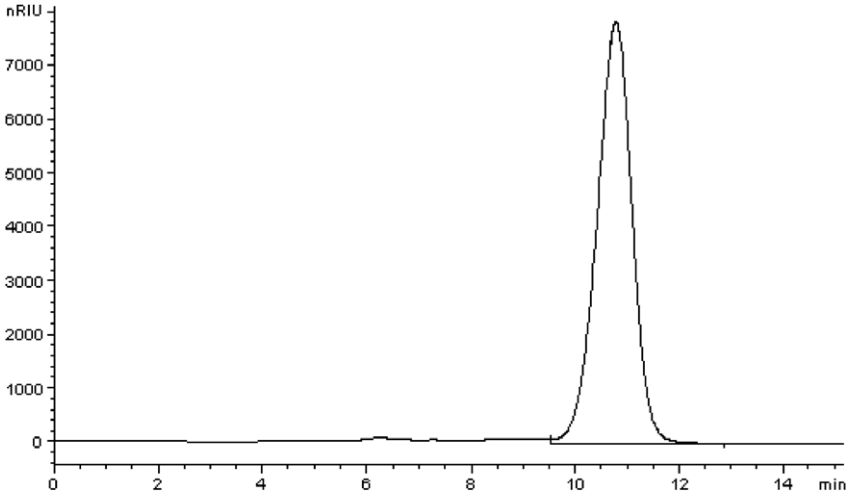

Fig. 1. Profile of FVP2 in HPGPC, eluted with H_2O at 1.0 mL/min.

Analysis of monosaccharide composition of FVP2, determined by TLC and GC analysis. The results showed that glucose is the only component. The big magnitude ($[\alpha]_D^{20}$ +160.19°) of its positive specific rotation and the characteristic IR absorption band at 843 cm^{-1} suggested the α-configuration of the glucosyl motif (Mathlouthi & Koenig, 1986). The optical rotations of FVP2 hydrolyzate was $[\alpha]_D^{20}$ +52.0° (*c* 0.2, H_2O), indicated the D-configuration of the glucosyl residues (Hara, Kiho, & Ukai, 1983; Liu et al., 2007). This result was in agreement with previous observation, that the bands at 762 and 929 cm^{-1} in the IR spectrum as the hallmark of D-glucopyranosyl residues in the polysaccharide (Zhang, 1999).

Every sugar residue of FVP2 consumes 1.132 mol periodate and librates 0.110 mol formic acid. The oxidized product was further undergone Smith degradation with the release of erythritol and trace of glycerol detected by TLC. The production of erythritol is the sign of (1→4)-linkages in the native glucan. The glycerin is from the oxidized branches of terminal glucosyl residues substituted at C-6 of the glucose residues. FVP2 was partially hydrolyzed with 0.1 M TFA. After periodate oxidation and Smith degradation, only erythritol was found by GC, indicating FVP2 was a polysaccharide with (1→4)-linked backbone. Only D-glucose was detected in the dialysate fraction, which confirmed the presence of single glucosyl groups as side chains.

Methylation analysis of FVP2 reveals the presence of three types of glucose derivatives as partially methylated alditol acetates (Table 1), 2,3,4,6-tetra-*O*-methyl-, 2,3,6-tri-*O*-methyl- and 2,3-di-*O*-methyl-derivatives in a close molar ratio of 1:6:1. All these results mean that the polysaccharide FVP2 is a glucan with (1→4)-linked backbone and (1→6)-linked branches. This pattern of linkage is also in accordance with the results by periodate oxidation and Smith degradation.

NMR spectroscopy was utilized to obtain the structural characterization of FVP2. The 500 MHz ^1H NMR spectrum of FVP2 (Fig. 2) had three anomeric protons at δ 5.47, 5.43 and 5.05 ppm, which are assigned as α-D-Glc*p*-(1→ (Residue A),→4)-α-D-Glc*p*-(1→) (Residue B) and α-D-Glc*p*-(1→4,1→6) (Residue C), respectively. Consistent with presence of an IR band 843 cm^{-1}, this confirms that the sugar residues are linked α-glycosidically (Perepelov et al., 2000). Chemical shifts from 3.4 to 4.0 ppm are designated as the protons of carbons C-2 to C-6 on glycodic ring (Chauveau, Talaga, Wieruszeski, Strecker, & Chavant, 1996; Wu et al., 2006). The α-configuration of the D-glucosyl groups is again conformed by the presence of three anomeric peaks, in the regions of δ 102.80, 102.43 and 101.57 ppm from ^{13}C NMR spectrum of FVP2 (Fig. 3). And branchings of C-6 are demonstrated by the signals of *O*-substituted C-6 at δ 72.19 and unsubstituted C-6 at δ 63.38 ppm. Predominant signal of latter, together with the typical signal of *O*-substituted C-4 at δ 79.80, support the results from chemical analysis, the high proportion of α-D-(1→4)-linkages in the linear arrangement. The multiple and broad signals at δ 79.80 could be ascribed to the pres-

Table 1
GC–MS of alditol acetate derivatives from the methylated product of FVP2

Methylated sugars (as alditol acetates)[a]	Molar ratio	Relative retention time[b]	MS main fragments (*m*/*z*)	Linkages indicated
2,3,4,6-Tetra-*O*-Me-Glc	1	1.00	43,45,71,87,101,117,129,145,161,205	Glc-(1→
2,3,6-Tri-*O*-Me-Glc	6	1.25	43,45,87,99,101,113,117,233	→4)-Glc-(1→
2,3-Di-*O*-Me-Glc	1	1.47	43,101,117,261	→4,6)-Glc-(1→

[a] 2,3,4,6-Tetra-*O*-Me-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucose, etc.
[b] Relative retention times of the corresponding alditol acetate derivates compared to 2,3,4,6-tetra-*O*-methyl-glucose.

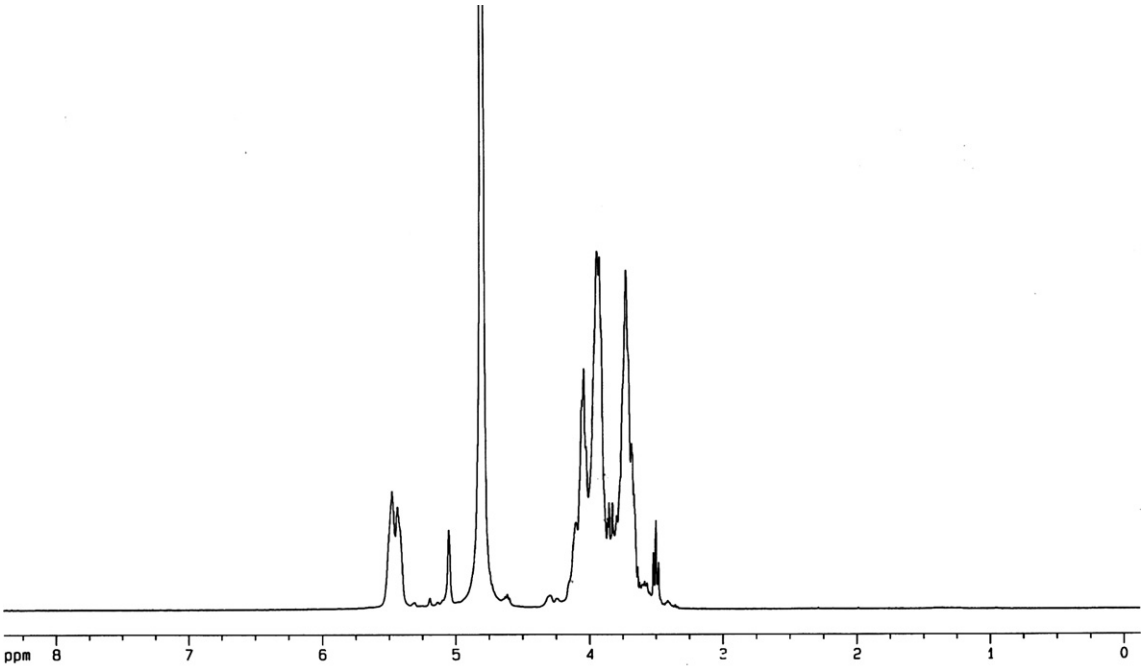

Fig. 2. ^{1}H NMR spectrum of FVP2 isolated from *Flammulina velutipes* mycelium recorded in D_2O.

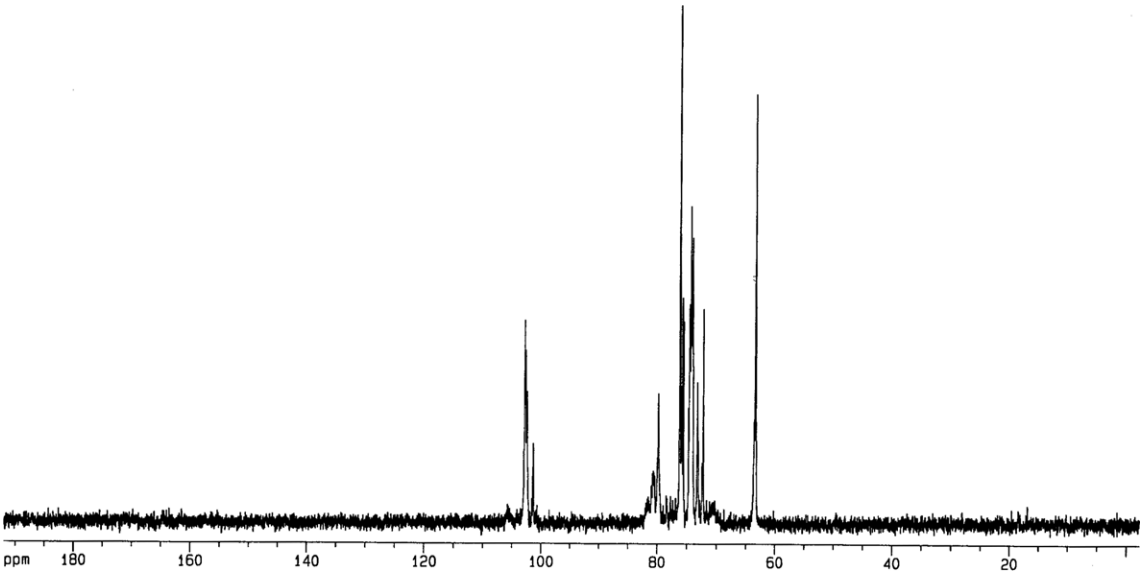

Fig. 3. ^{13}C NMR spectrum of FVP2 isolated from *Flammulina velutipes* mycelium recorded in D_2O.

ence, in the FVP2, of linear α-D-(1→4), branched α-D-(1→4,1→6), and terminal α-D-glucopyranosyl residues. The atom signals of glucosyl ring carbon at δ 75.73, 76.14, 74.35, 75.56, 63.38 correspond, respectively, to C-2, C-3, C-4, C-5, C-6 of Residue A (Agrawal, 1992; Muldoon et al., 2001). The ^{13}C NMR spectra of FVP2 were assigned according to 1D ^{13}C and HSQC (not shown) NMR data (Table 2). From the HSQC spectrum, C-6 correlate with two protons (Residue C: δ 3.73, 3.93; Residue A and B: δ 3.8–4.0). C-1 also correlate with two protons (Residue C: δ 5.05; Residue A and B: δ 5.4–5.5), which corresponds with results from the ^{1}H NMR spectrum. C-2, C-3, C-4, C-5 each correlate with one proton. The other proton signals (H_2–H_5) of FVP2 were not assigned due to overlapping peaks. All these resonances indicates the branched α-(1→4)-glucan structure of the native FVP2. Taken together, the putative structure of FVP2 can be proposed as Fig. 4.

3.2. Biological activity on hepatocytes

The effect of FVP2 on the hepatocytes was tested *in vitro* as described in Section 2. FVP2 could significantly stimulate the growth of primary hepatocytes at the dose of

Table 2
^{13}C NMR chemical shifts of FVP2 in D_2O

Residue	Chemical shifts (ppm)[a]					
	C-1	C-2	C-3	C-4	C-5	C-6
A, α-D-Glc*p*-(1→	101.57	75.73	76.14	74.35	75.56	63.38
B, →4)-α-D-Glc*p*-(1→	102.80	74.03	76.14	79.80	73.23	63.38
C, →4)-α-D-Glc*p*-(1→6↑	102.43	74.19	76.14	79.80	73.23	72.19

[a] In ppm downfield relative to the signal for DSS.

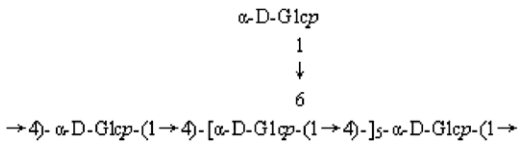

Fig. 4. Putative structure of polysaccharide FVP2 isolated from *Flammulina velutipes* mycelium.

25–200 μg/mL (Table 3). The liver injury animal model induced by CCL_4 is the common model representing the pathological progress of acute liver disease. The results show that FVP2 could protect hepatocytes against CCl_4 through preventing the release of intracellular ALT (Table 4). Compared with the control group, both the middle and high dosage of FVP2, significant decrease in ALT is observed, suggesting its protective effects on intoxicated hepatocytes induced by CCl_4. As proposed by Boll et al. (Boll, Weber, Becker, & Stampfl, 2001), the possible mechanisms involve the prevention of the production of $CCl_3^{\cdot}$ radical induced by CCl_4, the initiation of lipid peroxidation and the leakage of intracellular ALT.

Highly structural diversity of polysaccharides offers the highest capacity of carrying biological information. The monosaccharide units of polysaccharides can interconnect with each other at multiple points to form various branched or linear structures. Such structural variability of polysaccharides provides the necessary flexibility to achieve the precise regulatory mechanisms of cell–cell interactions in higher organisms. Lots of polysaccharides can significantly enhance the immune system. As a result, anti-tumor activity of these polysaccharides is believed to be the results of stimulation of host immune system rather than direct anti-cytotoxicity. For example, mushroom polysaccharides appear to be well tolerated and compatible with chemotherapy and radiation therapy. The exact molecular mechanisms of specific immune modulation by mushroom polysaccharides are unknown yet. It is possible because the binding of receptors with these polymers triggers specific downstream events in the targeted cells (Daba & Ezeronye, 2003).

Table 3
Stimulatory effect of FVP2 on growth of primary hepatocytes

Group	Dosage (μg/mL)	OD_{570}
Control		0.068 ± 0.008
FVP2	200	0.158 ± 0.013[a]
	100	0.146 ± 0.011[a]
	50	0.120 ± 0.016[a]
	25	0.100 ± 0.016[a]
HGF	200	0.306 ± 0.033[a]

Data are reported as means ± SD ($n = 5$).
[a] $P < 0.01$ vs. control.

Table 4
Protective effect of FVP2 on the primary hepatocytes damaged by CCl_4

Group	Dosage (μg/mL)	OD_{505}	$ALT_{(cal's\ unit)}$
Control		0.044 ± 0.002	12.18 ± 0.78
Model		0.083 ±0.003	25.86 ± 1.00[a]
FVP2	200	0.059 ± 0.004	17.55 ± 1.53[b]
	100	0.063 ± 0.002	18.67 ± 0.84[b]
	50	0.074 ± 0.002	22.51 ± 0.80[c]
	25	0.080 ± 0.003	24.60 ± 1.12
HGF	200	0.062 ± 0.002	18.46 ± 0.78[b]

Data are reported as means ± SD ($n = 5$).
[a] $P < 0.01$ vs. control.
[b] $P < 0.01$.
[c] $P < 0.05$ vs. model.

Fungus polysaccharides are regarded as biological response modifiers (BRM) (Leung et al., 2006). Most are relatively safe and impose no additional stress on the body, but help the body adapt to various environments and biological stresses. Almost all of the major systems of the body, including nervous, hormonal and immune systems as well as regulatory functions response well to fungus polysaccharides. The polysaccharides from fungus do not inhibit cancer cells directly, but display anti-tumor effects through activating different immune responses of the host. More than 50 mushrooms species have been found possess potential immunoceuticals and exhibit anticancer activity *in vitro* or in animal models (Daba & Ezeronye, 2003). Some of these polysaccharides that have been studied clinically in human cancers, including Lentinan, Schizophyllan, Active hexose correlated compounds (AHCC), Grifolan, polysaccharide-K and polysaccharide-P (Zhang et al., 2007). Lentinan has been proved successful to prolong the overall survival of cancer patients, especially those with gastric and colorectal carcinomas. Our study here suggests that *F. velutipes* polysaccharide has the protective effect on hepatocytes. The study of mechanism, compared with the known mechanisms will be our further direction.

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