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Chemical structure of one low molecular weight and water-soluble polysaccharide (EFP-W1) from the roots of *Euphorbia fischeriana*

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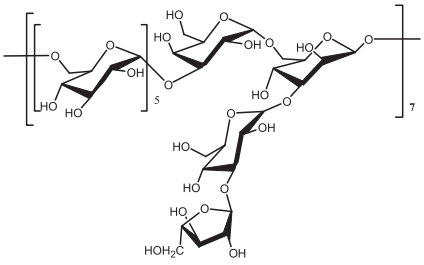
ABSTRACT

A water-soluble protein-bound polysaccharide (EFP-W1) was fractioned from roots of *Euphorbia fischeriana* and purified by gel-filtration chromatography. Its primary structural features were characterized by partial acid hydrolysis, Smith degradation-periodate oxidation, methylation analysis, high performance size exclusion chromatography (HPSEC), fourier transform infrared spectroscopy (FTIR), gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS) analysis and nuclear magnetic resonance spectroscopy (NMR). The data obtained indicated that EFP-W1 contains about 91% of carbohydrate content, which was mainly composed of glucose (Glc), galactose (Gal), mannose (Man), arabinose (Ara) in a molar ratio of 6:1:1:1. Its average molecular weight was about 11,230 Da. The structure of the repeating unit of the polysaccharide was elucidated as follows:



1. Introduction

Euphorbia fischeriana Steud (Euphorbiaceae) is a perennial herbaceous plant distributed widely in northeast mainland of China. The dried plant roots, named "lang-du" in traditional Chinese medicine, are used as a remedy for the treatment of a wide range of ailments, including edema, indigestion, as well as liver and lung cancers (Wang et al., 2006). Chemical investigation has led to the isolation of sterols, triterpenes, tannins, and a number of diterpenes compounds from this plant. In particular, diterpenes were thought to be the main bioactive compounds responsible for their significant antitumor activity (Wu et al., 2009). Wang et al. (2009) proposed that the natural compound 17-Hydroxy-jolkinolide B (HJB) is a promising anticancer drug candidate as a potent signal transducer and activator of transcription 3 (STAT3) signaling inhibitor. However there is not any information published on the polysaccharide from the roots of *E. fischeriana*. Nowadays plant polysaccharides had been drawn more attentions by researchers on their antitumor and immunological bioactivities for people. As a prerequisite to better understand its antitumor activity and underlying action mechanism, in this study we successfully purified and characterized one water-soluble polysaccharide from the roots of *E. fischeriana*.

2. Materials and methods

2.1. Materials

DEAE Sepharose Fast Flow, Sephadex G-100, and Sephadex G-25 were purchased from Amersham (Sweden). D-Glucose was form Amresco Inc. T-series dextran was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

2.2. General methods

Total sugar content of polysaccharide was quantified by the phenol–sulfuric acid method, with glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acid contents

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were determined by measuring the absorbance at 525 nm using the meta-hydroxybiphenyl colourimetric procedure and with D-glucuronic acid as the standard (Blumenkrantz & Asboe-Hansen, 1973). Protein in the polysaccharides was determined according to the Bradford's method (Sedmark & Grossberg, 1979), with bovine serum albumin as the standard. On the basis of the sulfate content, the sulfate content was determined by barium chloride–gelatin assay (Sun, Liang, Cai, et al., 2009; Sun, Liang, Zhang, Tong, & Liu, 2009). Infrared spectra of polysaccharides were recorded on SPECORD IR spectrometer in a range of 400–4000 cm^{-1}. Samples were dried at 35–44 °C in vacuum over P_2O_5 for 48 h prior to making pellet with KBr powder. UV–Vis absorption spectra were recorded with a UV–Vis spectrophotometer (Model SP-752, China). Gaschromatography (GC) was used for identification and quantification of sugar, which was performed on a Shimadzu GC-14C instrument (Japan) equipped with a DB-1 capillary column (30 m × 0.25 mm × 0.25 μm) and flame-ionization detector (FID). The column temperature was kept at 120 °C for 2 min and increased to 250 °C (maintained for 3 min) at a rate of 8 °C/min. The injector and detector heater temperature were 250 and 300 °C, respectively. The rate of N_2 carrier gas was1.2 ml/min. Gas chromatography–mass spectrometry (GC–MS) was finished on a Shimadzu QP-2010 instrument (Japan) with an HP-5MS quartz capillary column (30 m × 0.25 mm × 0.25 μm). GE Healthcare's ÄKTA Explore 100 purification system was applied to the process of polysaccharide fraction, which was equipped with UV-900 monitor, a P-900 series pump, M-925 mixer, pH/C-900 detector, Frac-950 fraction collector, A-900 auto-sampler and various kinds of columns. Dialysis was carried out using tubing with a Mw cut-off of 500 Da (for globular proteins). All gel chromatography was monitored with phenol–sulfuric acid method.

2.3. Isolation and purification of EFP-W1

The powdered roots of *E. fischeriana* (0.5 kg) were extracted with 95% ethanol (5000 ml, × 3) at 75 °C for 6 h under reflux to remove lipid. The residue was then extracted with distilled water (5000 ml) at 75 °C for 3 times and 3 h for each time. After centrifugation (1700 × *g* for 15 min), the supernatant was concentrated to one tenth of the volume, and precipitated with 4 vol of 95% ethanol at 4 °C for 24 h. The precipitate collected by centrifugation was deproteinated by proteinase digestion and the Sevag method (Sun & Liu, 2009), followed by exhaustive dialysis with water for 48 h. Then the concentrated dialyzate was precipitated with 4 vol of 95% EtOH at 4 °C for 24 h. The precipitate was washed with absolute ethanol, acetone, and ether. The washed precipitate was the crude polysaccharide (CEFP, 15.9 g).

The CEFP was purified on an ÄKTA explore 100 purification system. The pretreated sample was applied to a DEAE Sepharose Fast Flow column (2.6 cm × 40 cm) equilibrated with distilled water. After loading with sample, the column was eluted with distilled water and then with stepwise gradient of NaCl aqueous solutions (0.1, 0.3, 0.5, and 1 M) at a flow rate of 4 ml/min. Different fractions were collected using the Frac-950. Total sugar content of each tube was measured at 490 nm by Dubois's method, and protein absorption at 280 nm was recorded for each fraction. The water-eluted solution was separated into one fraction only and then purified further on a Sephadex G 100 column (2.6 cm × 100 cm) with 0.15 M NaCl at a flow rate of 1 ml/min, yielding only the EFP–W1 fraction (0.4 g), and then was applied to a Sephadex G-25 column (2.6 cm × 40 cm) to remove salts.

2.4. Determination of sugar composition, purity and molecular weight

GC was used for identification and quantification of the monosaccharide composition of polysaccharide. EFP-W1 was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. The monosaccharides were conventionally converted into the alditol acetates as described (Jones & Albersheim, 1972; Oades, 1967) and analyzed by GC as previously mentioned.

The homogeneity and molecular weight of EFP-W1 was evaluated and determined by high-performance size-exclusion chromatography (HPSEC) (Sun & Liu, 2009). An aliquot of 20 μL the sample solution (0.5%) was performed on a SHIMADZU HPLC system fitted with one TSK-G3000 PW_{XL} column (7.8 mm ID × 30.0 cm), eluted with 0.1 mol/L Na_2SO_4 solution at a flow rate of 0.5 ml/min and detected by a SHIMADZU refractive index detector (RAD-10A). The column was calibrated with T-series dextran (T-130 80, 50, 25, 10) as standards. The molecular weight of PCP-W1 was estimated by reference to the calibration curve made above.

2.5. Partial hydrolysis with acid

The EFP-W1 (100 mg) was hydrolyzed with 0.05 M TFA (3 ml), kept at 95 °C for 10 h, then centrifuged, the precipitate was sent to GC analysis. The supernatant was dialyzed with distilled water for 48 h, followed precipitated with ethanol. Precipitation in the sack, supernatant in the sack, and the fraction out of sack were dried and carried out for GC analysis as previously described (Sun et al., 2008).

2.6. Periodate oxidation and Smith degradation

The sample (25 mg), dissolved in 12.5 ml of distilled water, was mixed with 12.5 ml of 30 mM $NaIO_4$. After the mixture was kept in darkness for 48 h at 4 °C, an aliquot (0.1 ml) were withdrawn from the mixture at very 3–6 h and recorded in a spectrophotometer at 223 nm (Linker, Evans, & Impallomeni, 2001) after dilution 250-fold with distilled water. $NaIO_4$ consumption was calculated on the basis of the change of the absorbance at 223 nm. The solution of periodate product (2 ml) was used to assess the amount of formic acid by titration with 0.01 M sodium hydroxide, and the remnant was dialyzed extensively against water and distilled water for 24 h, respectively. The content inside was concentrated and reduced by $NaBH_4$ (60 mg) overnight, neutralized with 50% acetic acid, dialyzed, freeze-dried and analyzed using GC; others were hydrolyzed with 1 M sulfuric acid for 40 h at 25 °C, neutralized to pH 6.0 with $BaSO_4$, and filtered for analysis. The filtrate was dialyzed and the dialysate out of the sack was lyophilized for GC analysis. The content inside the sack was precipitated with ethanol result in two fractions, namely the supernatant and precipitate, which were also dried out for GC analysis after centrifugation.

2.7. Methylation analysis

The sample (20 mg) was methylated three times, according to Needs and Selvendran (1993). The methylated products were extracted by chloroform. The disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum confirmed that complete methylation. The methylated products were hydrolyzed with formic acid and 2 M TFA, then reduced with $NaBH_4$ for 24 h and acetylated with acetic anhydride–pyridine (1:1) at 100 °C for 2 h. The alditol acetates of the methylated sugars were analyzed by GC–MS.

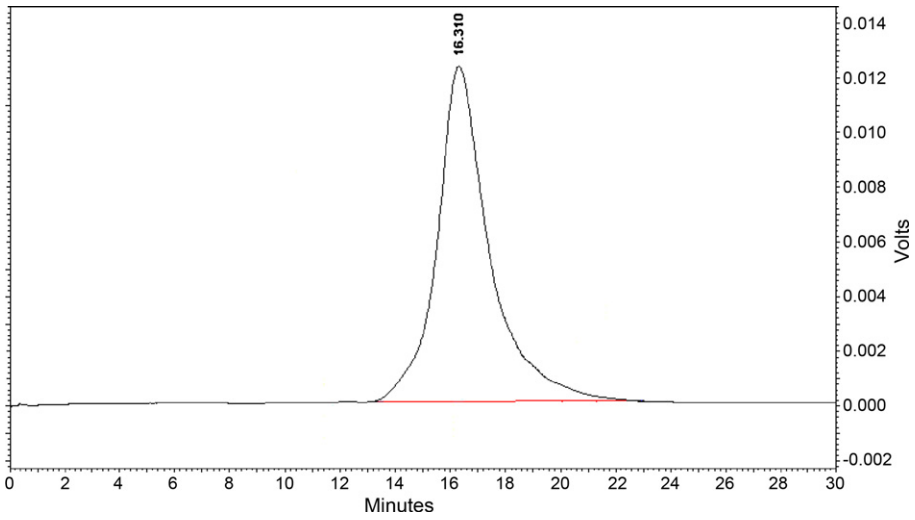

Fig. 1. HPSEC of EFP-W1.

2.8. NMR analysis

Deuterium-exchanged EFP-W1 (30 mg) was dissolved in deuteroxide (99.99% D, 0.55 ml) accompanied with ultrasonic wave processing for 30 min. Then the NMR spectrometer (Bruker AV-600) was used to perform the ^{1}H and ^{13}C analysis. Acetone was used as an external standard for the ^{13}C spectrum, and D_2O was used as internal standard for ^{1}H NMR spectrum (Sun, Li, Yang, Liu, & Kennedy, 2010).

The above methods applied in this paper are expressed in a conventional way as other papers published by our research group.

3. Results and discussion

3.1. Isolation, purification and chemicophysical properties of EFP-W1

After purification on ÄKTA Explore 100 purification system packed with DEAE Sepharose Fast Flow column and Sephadex G 100 column in succession, one homogeneous and purified polysaccharide was fractioned from the roots of *E. fischeriana*, as determined by HPSEC in Fig. 1. Its molecular weight was estimated to be about 11,230 Da.

The percentages of polysaccharide existed in EFP-W1 were determined by the phenol–sulfuric acid method to be 91%. In the UV spectrum of the EFP-W1, no absorbance peak at 280 nm and its negative response to the Bradford test confirmed absence of protein. Moreover there is small amount of 2% uronic acid and 0.24% sulfate radical present in EFP-W1 evaluated by the meta-hydroxydiphenyl colorimetric method and barium chloride–gelatin assay, respectively. The GC analysis indicated EFP-W1 mainly consisted Glc, Gal, Man and Ara in the proportion of 6:1:1:1

The EFP-W1 had IR bands at 1000–1150 cm^{-1}, 1400–1550 cm^{-1}, 2800–2900 cm^{-1}, and 3100–3500 cm^{-1}, which were distinctive absorptions of polysaccharides. The absorption band at 840 and 890 cm^{-1} confirmed the co-existence of α and β-glycosidic bond (Fig. 2), which was in good agreement with the results of NMR analysis.

3.2. Structural characterization of EFP-W1

The fully methylated product of EFP-W1 was hydrolyzed, converted, and analyzed by GC/MS. The results demonstrated there are total five liberations of 2,3,4,-Me_3-Glc*p* (Residue A: 1,6-linked Glc), 2,3,4,-Me_3-Gal*p* (Residue B: 1,6-linked Gal), 2,4-Me_2-Man*p* (Residue C: 1,3,6-linked Man), 2,4,6-Me_2-Glc*p* (Residue D:

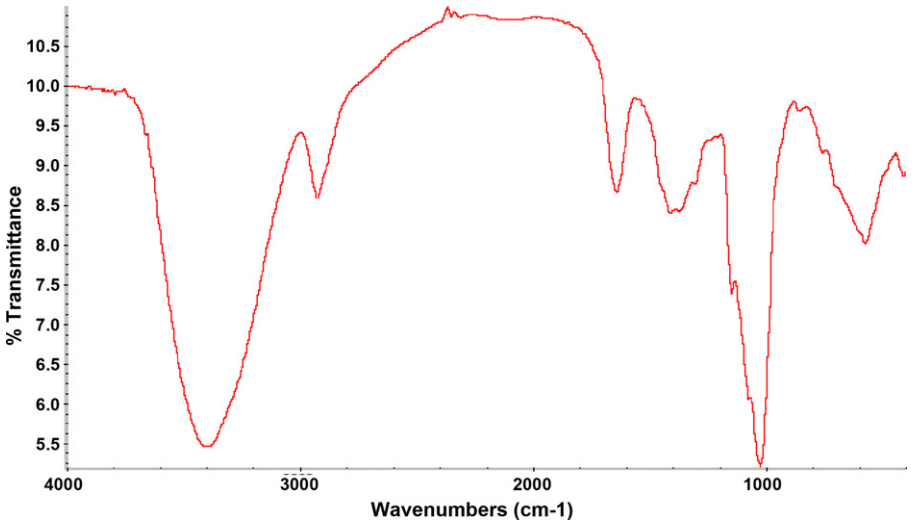

Fig. 2. The FTIR spectra of EFP-W1.

Table 1
GC–MS results of methylation analysis of EFP-W1.

Peak no.	Methylated sugar	Molar ratio	Linkage type
Residue A	2,3,4,-Me_3-Glc*p*	5	1,6-linked Glc
Residue B	2,3,4,-Me_3-Gal*p*	1	1,3-linked Gal
Residue C	2,4-Me_2-Man*p*	1	1,3,6-linked Man
Residue D	2,4,6-Me_2-Glc*p*	1	1,3-linked Glc
Residue E	2,3,5,-Me_3-Ara*f*	1	1-linked Ara

1,3-linked Glc) and 2,3,5,-Me_3-Ara*f* (Residue E: 1-linked Ara) in a relative molar ratio of 5:1:1:1:1 (Table 1). The main backbone chain of EFP-W1 was predominantly composed of Residue A, B and C, branched at O-3 position of Residue C with Residue D and E. In summary, those results from analysis of GC/MS were completely consistent with the observation of partial acid hydrolysis, periodate oxidation and smith degradation.

^{13}C NMR spectra of EFP-W1 were recorded for aqueous solutions in D_2O. The spectra are shown in Fig. 3. In the ^{13}C NMR spectrum of EFP-W1, the signals at 99.082, 99.248, 99.082, 101.36 and 106.394 ppm attributed to the anomeric carbons of Residue A, B, C, D, and E, respectively. The predominance of Residue A, together with the typical signal at d 99.082, supported the high proportion of 1, 6-linked-α-D-Glc*p* in EFP-W1. Peaks outside the C-1 region provided further information about the chemical structure. Peaks at 81.560 and 80.850 ppm in the spectrum were both assigned to C-3 of Residue B and D, respectively. Signal 83.527 ppm appeared in low magnetic field should be attributed to C-4 of Residue E. The chemical shifts from 59.936 to 83.527 ppm were assigned to C-2 to C-6 of glycosidic ring (Chauveau, Talaga, Wieruszeski, Strecker, & Chavant, 1996). Three signals appeared at high magnetic filed: 59.936 and 60.577 ppm seem to be C-6 singal of Residue B and D; 62.550 ppm seem to be C-5 of Residue E, because of no substitution on the C-6 or C-5 position for Residue B and D, or Residue E. On the contrary, peak 68.832 and 71.011 were assigned to substituted C-6 of Residue A and C, respectively. Fig. 4 showed the 600-MHz ^1H NMR spectrum of EFP-W1, the peaks at 5.336, 5.091, 4.904, 5.027 and 5.188 ppm were assigned to the H1 signals of Residue A, B,

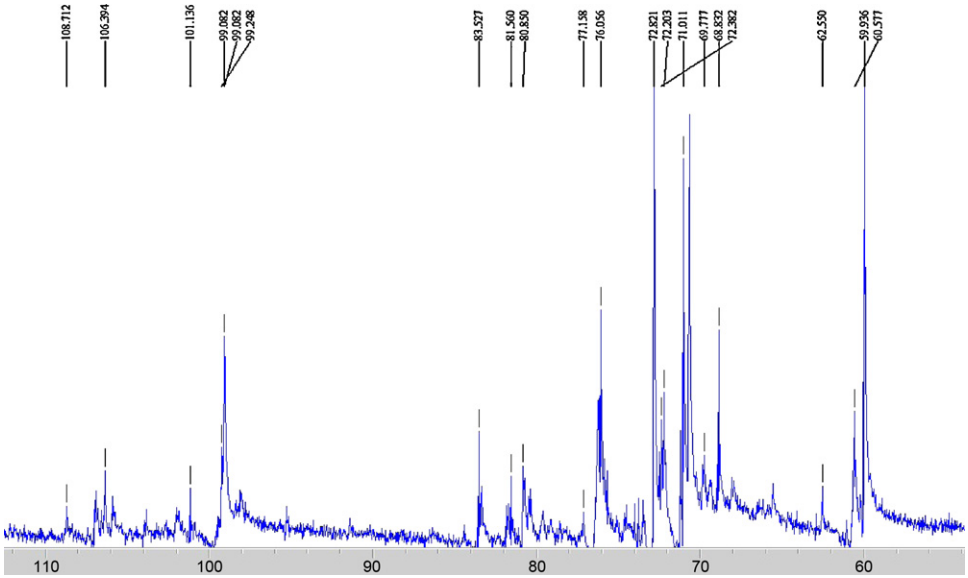

Fig. 3. ^{13}C NMR (150 M) spectra of EFP-W1.

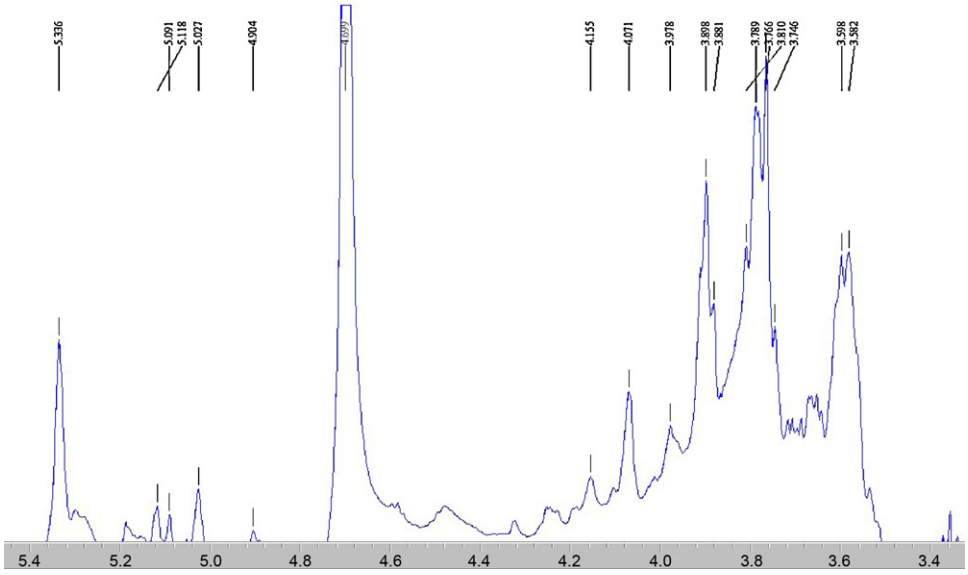

Fig. 4. ^1H NMR (600 M) spectra of EFP-W1.

C, D and E, respectively. The special anomeric carbons and their protons' chemical shift confirmed that sugar residues were linked α-glycosidically except for Residue C with a β-glycosidic bond, which agreed with co-presence of an IR band 840 and 890 cm^{-1} (Barker, Bourne, Stacey, & Whiffen, 1954). The other proton signals (H2–H5) of EFP-W1 were not assigned due to overlapping peaks. All the NMR chemical shifts were compared with the literature values (Dey et al., 2010; Habibi, Heyraud, Mahrouz, & Vignon, 2004; Katzenellenbogen et al., 2005; Ojha, Chandra, Ghosh, & Islam, 2010; Ovchinnikova et al., 2011; Perepelov et al., 2010; Sun, Li, & Liu, 2010; Sun, Liang, Cai, et al., 2009; Sun, Liang, Zhang, et al., 2009; Sun & Liu, 2008; Sun et al., 2008; Yang, Cisar, & Bush, 2011; Zhang, Zhang, Yang, & Liang, 2010).

4. Conclusion

From the above analysis, we elucidated that the structural feature of EFP-W1 from the roots of *E. fischeriana* had the following structure: the backbone consisted of 7 repeating units of [→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)- α-D-Gal*p*-(1→3,6)-β-D-Man*p*-(1→]; the side chain [→1)- α-D-Glc*p*-(3→1)- α-L-Ara*f*] was attached to the backbone through O-3 of Man residues. The further detailed structure elucidation would continue in our later research.

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