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

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Abstract—A new heteropolysaccharide (HEP-1) was isolated from the fruiting bodies of *Hericium erinaceus*. It was estimated to have a molecular weight of 1.8×10^4 da and showed $[\alpha]_D^{20}$ +129 (*c* 0.295, H_2O). HEP-1 is composed of rhamnose, galactose, and glucose in the ratio of 1.19:3.81:1.00. Its structural features were investigated using composition analysis, methylation analysis, partial hydrolysis, and IR and NMR spectroscopy. The results showed that HEP-1 has a (1→6)-linked α-D-galactopyranosyl backbone with branches that are composed of rhamnose and glucose attached to O-2.

Keywords: *Hericium erinaceus*; Rhamnoglucogalactan; Polysaccharide; Chemical structure

1. Introduction

Hou Tou Gu (bear's head) is the fruiting body of *Hericium erinaceus* (Bull.) Pers., which is used as an edible and medicinal fungus in China. As a Chinese traditional medicine, it is used for the treatment of neurasthenic gastritis and gastroduodenal ulcer, among other ailments. The fruiting body was reported to contain various constituents such as hericenone, erinacine, terpenoids, lectins, proteins, lipids, and polysaccharides. Some polysaccharide and polypeptide fractions of *H. erinaceus* were reported to exhibit antitumor activity.[1] We have isolated five major polysaccharide fractions from this fruiting body, and in this communication, we report on the structural investigation on HEP-1, a heteropolysaccharide isolated from a water extract.

2. Results and discussion

On HPGPC (high performance gel-permeation chromatography), HEP-1 showed one symmetrical peak, indicating its homogeneity. According to the retention time, its molecular weight was estimated to be 1.8×10^4 da, and it showed an $[\alpha]_D^{20}$ +129 (*c* 0.295, H_2O). The *o*-phthalic acid method detected no uronic acid.[2] After hydrolysis with 2 M TFA, TLC analysis showed the completeness of hydrolysis and no pink spot characteristic of uronic acids.[3] The hydrolysate was transformed into the alditol acetates after reduction and acetylation. GLC analysis showed that HEP-1 was composed of rhamnose, galactose, and glucose with L-, D-, D-configurations, respectively, in a molar ratio of 1.19:3.81:1.00.

The permethylated polysaccharide was hydrolyzed, reduced, and acetylated, then subjected to GC–MS (see Table 1). Methylation analysis of HEP-1 showed that the galactosyl residues are mainly (1→6)-linked with a small number of 1,2,6-linked Gal units. The rhamnosyl residues are completely distributed at nonreducing terminals. The glucosyl residues are mainly 1,2,6-linked. These results indicated that this polysaccharide possibly contains a backbone consisting of (1→6)-linked galactosyl residues. It remains to be determined whether the glucosyl residues are positioned in the backbone or in side chains.

After the first hydrolysis in 0.05 M TFA, HEP-1 gave a dialysate that was found to contain mainly rhamnose. The retentate, HEP-1-SP1, was shown as a

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Table 1. Results of the methylation analysis of HEP-1 by GC–MS

Methylation positions	Linkages	Molar ratios	Major mass fragments (*m/z*)
2,3,4-Me_3-Rha	1-linked Rha*p*	19.5	72, 81, 101, 115, 117, 131, 161, 175
2,3,4,6-Me_4-Glc	1-linked Glc*p*	1.1	45, 71, 87, 101, 117, 129, 145, 161, 205
2,3,4-Me_3-Glc	1,6-linked Glc*p*	1.1	59, 71, 87, 99, 101, 117, 129, 161, 173, 189, 233
3,4-Me_2-Glc	1,2,6-linked Glc*p*	14.3	71, 87, 99, 129, 189
2,3,4,6-Me_4-Gal	1-linked Gal*p*	0.9	45, 71, 87, 101, 117, 129, 145, 161, 205
2,3,4-Me_3-Gal	1,6-linked Gal*p*	53.5	59, 71, 87, 99, 101, 117, 129, 161, 173, 189, 233
3,4-Me_2-Gal	1,2,6-linked Gal*p*	9.7	71, 87, 99, 129, 189

homogeneous polysaccharide with a molecular weight of 1.1×10^4 by HPGPC that was composed of Rha, Gal, Glc in the ratio of 0.32:3.94:1.00. In comparison with the composition of the native polysaccharide (1.19: 3.81:1.00), the proportion of rhamnose significantly decreased, while the ratio of galactose to glucose remained almost unchanged. Methylation analysis of HEP-1-SP1 (Table 2) showed that with the reduction of Rha terminals, 1,2,6-linked Glc*p* disappeared along with the increasing 1,6-linked Glc*p*, indicating the released rhamnose units were originally linked to O-2 of 1,2,6-branched-Glc*p*. Hydrolysis of HEP-1-SP1 gave a dialysate corresponding to a monosaccharide, which was also shown to be mainly rhamnose. HEP-1-SP2 contains Rha, Gal and Glc, in the ratio of 0.11:2.17:1.00 and showed a molecular weight of 7200, while HEP-1-SP3 was shown to contain only galactose and had a molecular weight of 2800. The significant reduction of molecular weights indicated they were derived from the depolymerization of HEP-1-SP1, rather than from a mixture of a glucogalactan and a galactan. These results also indicated that the glucosyl residues are probably not homogeneously distributed in HEP-1-SP1. Thus the cleavage of the inner chain gave glucosyl-rich HEP-1-SP2 and HEP-1-SP3 in which glucose was absent. Methylation analysis (Table 2) showed that HEP-1-SP3 is a linear (1→6)-linked galactan with an average degree of polymerization (DP) of 14, and HEP-1-SP2 contains mainly (1→6)-linked glucosyl and galactosyl residues, except for a small number of terminal units and 1,2,6-Gal*p* linkages. This result indicated that HEP-1-SP2 probably has an α-(1→6)-D-galactan backbone, with (1→6)-linked β-D-glucose as the side chains attached to O-2 of the backbone. However, it does not exclude the possibility that (1→6)-linked Gal*p* and (1→6)-linked Glc*p* coexist alternatively in the backbone. To settle such a question, HEP-1-SP2 was further hydrolyzed. The supernatant yielded an oligosaccharide fraction (DP > 3) composed only of glucose. HEP-1-SP4 was recovered as the precipitate and consisted mainly of (1→6)-linked galactosyl residues as shown in Table 2, indicating that glucose and galactose are distributed in the side chains and backbone, respectively. After partial hydrolysis, the side chains are released as a glucosyl oligomer, and the backbone has thus evolved into a degraded galactan, HEP-1-SP4.

In the anomeric region of the ^{1}H NMR spectrum (Fig. 1a) of HEP-1, three signals occurred at δ 4.50, δ 4.95, and δ 5.05 ppm. Their assignments were compared with the literature values.[4] The strongest signal at δ 4.95 ppm should arise from D-Gal due to its high content, indicating an α-anomeric configuration. The weakest one at δ 4.50 ppm was assigned to D-Glc adopting a β-anomeric configuration. Thus the signal at δ 5.05 ppm could only be assigned to α-L-Rha. Similarly, the three anomeric signals in the ^{13}C NMR spectrum (Fig. 1b) of HEP-1 were assigned as follows: δ 99.96 (α-D-Gal), δ 106.11 (β-D-Glc), δ 103.59 (α-L-Rha). The assignment of δ 103.59 ppm to α-L-Rha was corroborated by the absence of this signal in the ^{13}C NMR spectrum of HEP-1-SP2, in which rhamnose has been mostly removed. The high-field signal at δ 17.78 ppm was from C-6 of rhamnose, and the corresponding ^{1}H signal was at δ 1.19 ppm. In the DEPT spectrum two CH_2 resonances at δ 68.57 and δ 68.87 ppm were assigned to C-6 of galactose and glucose, respectively, indicating their substitution at

Table 2. Methylation analysis of HEP-1-derived products by GC–MS

Methylation positions	Linkages	Molar ratios			
		HEP-1-SP1	HEP-1-SP2	HEP-1-SP3	HEP-1-SP4
2,3,4-Me_3-Rha	1-linked Rha	6.3	2.3	—	
2,3,4,6-Me_4-Glc	1-linked Glc	1.4	5.1	—	
2,3,4-Me_3-Glc	1,6-linked Glc	17.7	25.2	—	
3,4-Me_2-Glc	1,2,6-linked Glc	—	—	—	
2,3,4,6-Me_4-Gal	1-linked Gal	1.6	5.1	6.9	8.2
2,3,4-Me_3-Gal	1,6-linked Gal	63.4	44.0	93.1	91.8
3,4-Me_2-Gal	1,2,6-linked Gal	9.6	17.5	—	

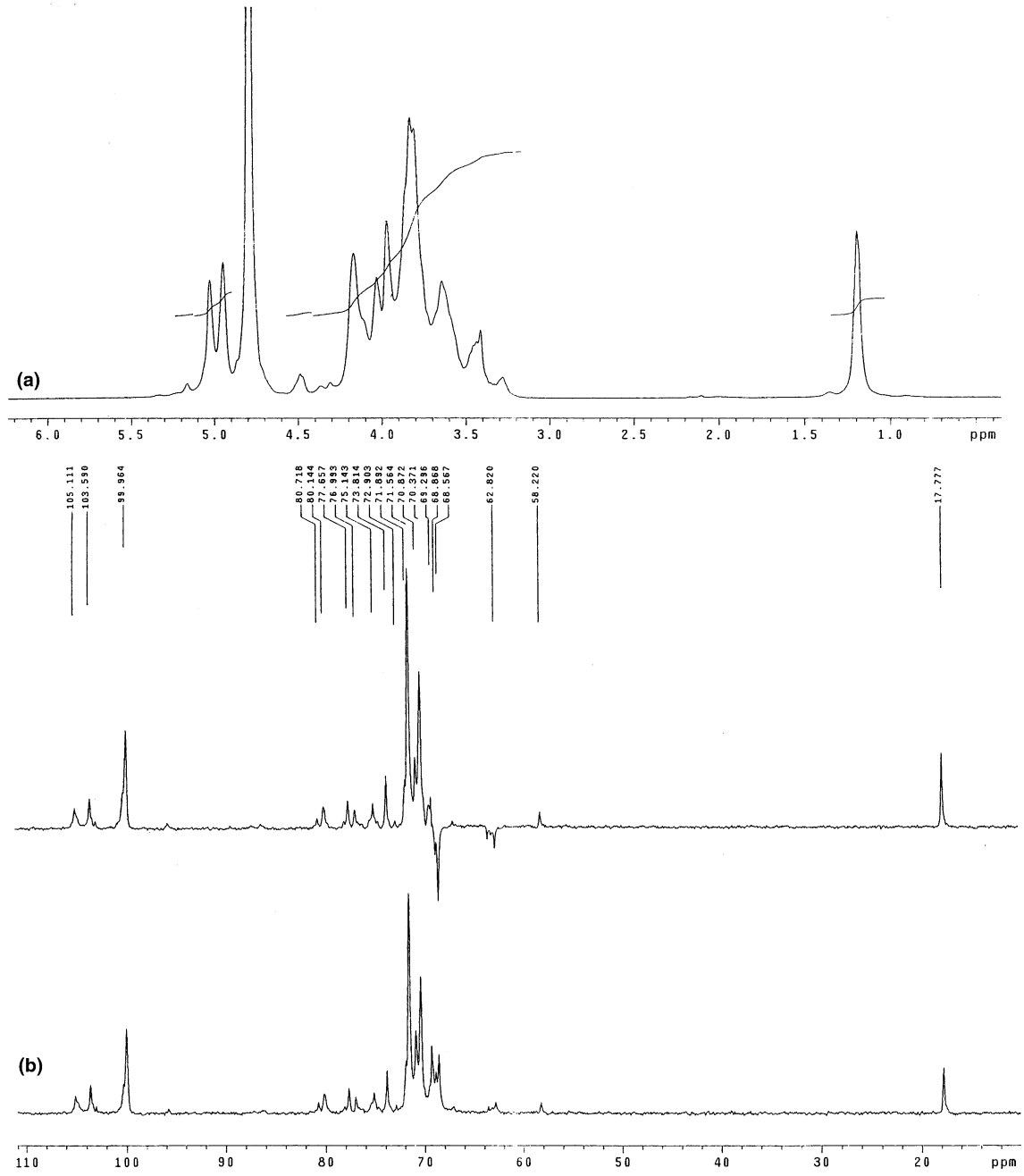

Figure 1. ^{1}H and ^{13}C NMR spectra of native HEP-1 at 400 MHz (in D_2O).

C-6.[5] The weak resonance of δ 62.82 ppm indicated that almost all of the C-6 of galactosyl and glucosyl residues were substituted.

Taken together, it was proposed that HEP-1 consists of an α-(1→6)-D-galactopyranan backbone. To some units of the backbone at O-2 are attached branches consisting of mainly of β-(1→6)-D-oligoglucosyl units and a minor terminal rhamnose residue. The rhamnoglucosyl branches are not homogeneously distributed on the backbone, with one end highly branched and the other end with almost no branching. Most glucosyl residues in the branches have O-2-substituted rhamnosyl as terminals, and the average DP of the branches is 3.7.

In most fungi polysaccharides have been reported to be the major component of the cell wall and intercellular matrix, involving β-(1→3)- and β-(1→6)-D-glucan and (1→6)-α-D-mannan, with the latter mainly found in yeast cell walls.[6] Their biological effects have been widely studied for their immunostimulating and antitumor activities.[7] A structure like HEP-1 containing a (1→6)-linked α-D-galactan backbone and branches

composed of glucose and rhamnose has not been previously reported, so HEP-1 is a novel fungal polysaccharide. It remains unclear if it is a specific component characteristic of *H. erinaceus*, and, furthermore, its physiological function awaits further studies.

3. Experimental

3.1. Materials

The fruiting bodies of *H. erinaceus* were cultivated in Li Shui, Zhejiang Province and provided as dried fruiting bodies by Qing Yuan Fang Ge Medicinal and Health Product Co. Monosaccharide standards (glucose, galactose, rhamnose, mannose, arabinose, and xylose) are all Fluka products, and the standards for MW determination, Dextran T-2000, T-700, T-580, T-500, T-80, T-70, T-40, T-11, and T-9.3 were purchased from Pharmacia Co. Sodium borohydride, trifluroacetic acid (TFA), (+)-2-butanol, and DC-Alufolien cellulose TLC plates were from Merck-Schuchardt Co. Other reagents were analytical grade unless otherwise claimed.

3.2. General methods

The ^{1}H, ^{13}C NMR spectra were recorded on a Varian DMX-400 spectrometer at 300 K, using Me_4Si as the external reference. A polarization transfer pulse of 135° was used in the DEPT experiments. IR spectra were recorded on a Perkin–Elmer 591B spectrophotometer. GLC was performed on a Shimadzu GC-14B chromatograph equipped with a packed glass column (2.5 m × 3 mm) of 3% OV-225/AW-DMCS-Chromosorb W (80–100 mesh) and a FID detector, column temp: 210 °C. GC–MS were performed on Shimadzu QP-5050A apparatus. The optical rotation was determined on a WZZ-1S automatic polarimeter (Shanghai Physical Optics Instrument Co.). Evaporation under vacuum was performed on a Büchi 461 rotary evaporator with a working temperature of <40 °C.

3.3. Extraction and isolation

The dried fruiting bodies (1.5 kg) of *H. erinaceus* were refluxed with 95% EtOH in a Soxhlet extractor for 6 h to remove lipids, and the mixture was then filtered. The residue was dried in air and then extracted with boiling water three times (6 h for each), then filtered. The filtrate was concentrated, dialyzed, and centrifuged to remove insoluble material. The supernatant was diluted with 3 vols of 95% EtOH. The precipitate was recovered by centrifugation and washed successively with absolute EtOH and acetone, then dried in vacuo at 45 °C, yielding the crude polysaccharide, CPW (40.8 g, 2.7%). CPW (20 g), after deproteination by the Sevag method,[8] was applied in several runs to a DEAE-cellulose column (4.5 cm × 50 cm, Cl^-) and eluted stepwise with distilled water, then with 0.1, 0.2, 0.4, and 0.8 M aq NaCl. The eluate was monitored by the phenol–sufuric acid method.[9] The water eluate was concentrated, dialyzed, and freeze dried, then separated on a Sephadex G-200 column (2.6 cm × 60 cm), that was equilibrated and eluted with 0.2 M NaCl. HEP-1 was obtained as the major fraction (yield, 1.08 g, 0.14% for the starting material).

3.4. Purity and molecular weight determination

The purity and molecular weight were determined by the HPGPC method,[10] which was performed on a Waters HPLC system, including a Model 515 pump, two serially linked Ultrahydrogel^{TM} 2000 and 500 columns, a Waters 2410 RI detector, a Waters 2487 dual wavelength UV detector, and an on-line degaser. The data were processed by GPC processing software (Millennium^{32} version). The mobile phase was 0.003 M NaOAc, and the flow rate was 0.5 mL/min. The sample (2 mg) was dissolved in the mobile phase (0.2 mL) and centrifuged (10,000 rpm, 3 min). A 20-μL sample was injected in each run. The molecular weight was estimated by reference to the calibration curve made from a Dextran T-series standard of known molecular weight (T-2000, T-700, T-580, T-500, T-80, T-70, T-40, T-11, and T-9.3).

3.5. NMR spectroscopy

The dried polysaccharide (~50–60 mg) was put in a 5-mm NMR tube and dissolved in 0.5 mL D_2O (degraded polysaccharides, HEP-1-SP1, and HEP-1-SP2 dissolved in D_2O–NaOD).

3.6. Composition analysis

The polysaccharide (3 mg) was dissolved in 2 M TFA (4 mL) and hydrolyzed in a sealed tube at 110 °C for 3 h. The hydrolysate was evaporated at reduced pressure to dryness with repeated addition of MeOH, then dissolved in 0.2 mL of distilled water and analyzed on a DC-Alufolien cellulose TLC plate (5:5:3:1 EtOAc–pyridine–H_2O–HOAc as the developing solvent) to check the completeness of hydrolysis and the presence of uronic acid. The plate was sprayed with aniline–*o*-phthalic acid reagent and heated at 100 °C for 10 min for visualization. The remaining hydrolysate was transformed into the corresponding alditol acetates, according to the previous procedure,[11] then subjected to GLC analysis. The absolute configurations of the monosaccharides were determined as described by Vliegenthart and co-workers using (+)-2-butanol.[12]

3.7. Methylation analysis

HEP-1 (9 mg) was methylated thrice according to the Ciucanu–Kerek method.[13] After complete methylation as shown by IR (Nujol), the permethylated polysaccharide was depolymerized in 90% HCO_2H at 100 °C for 3 h, then hydrolyzed in 2 M TFA at 100 °C for another 6 h. After $NaBH_4$ reduction and acetylation, the partially methylated alditol acetates were analyzed by GC–MS.

3.8. Graded hydrolysis

HEP-1 (200 mg) was first hydrolyzed in 0.05 M TFA at 100 °C for 1 h, then evaporated to dryness and dialyzed for 24 h. The dialysate was applied to a Sephadex G-10 column (80 cm × 2.6 cm) and eluted with distilled water. The retentate was freeze-dried to give the degraded polysaccharide, HEP-1-SP1 (163 mg). HEP-1-SP1 (100 mg) was further hydrolyzed in 0.05 M TFA at 100 °C for 1 h, then dialyzed. The dialysate was applied to a Sephadex G-10 column as above. A precipitation occurred in the nondialysate portion. After centrifugation and freeze-drying, the supernatant gave HEP-1-SP2 (45 mg), with the precipitate giving HEP-1-SP3 (30 mg). HEP-1-SP2 (30 mg) was treated in 0.1 M TFA for another 1 h at 100 °C. After evaporation under diminished pressure, the residue was dissolved in 1 mL of H_2O and precipitated with 3 vols of EtOH, giving HEP-1-SP4 (18 mg) as the precipitate. The supernatant was concentrated and applied to a Sephadex G-10 column. Methylation analyses were performed for HEP-1-SP1, HEP-1-SP2, HEP-1-SP3, and HEP-1-SP4, respectively. The graded hydrolysis-derived dialyzable fractions, after elution from the Sephadex G-10 column, were each subjected to composition analysis.

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