Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Research 341 (2006) 791–795
Carbohydrate RESEARCH

Note

A β-D-glucan isolated from the fruiting bodies of *Hericium erinaceus* and its aqueous conformation

Qun Dong,* Lian-meng Jia and Ji-nian Fang

Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Science, Graduate School, Chinese Academy of Sciences, 555 Zu Chong Zhi Road, Shanghai 201203, China

Received 17 October 2005; accepted 14 December 2005
Available online 3 February 2006

Abstract—HEP3, a β-D-glucan slightly soluble in water, was isolated from the alkaline extract of the fruiting bodies of *Hericium erinaceus*. Its chemical structure was investigated by methylation analysis, periodate oxidation, Smith degradation and by IR and NMR spectroscopy. It was shown to have a main chain composed of β-(1→3)-linked D-glucopyranosyl residues, with single unit glucosyl branches attached to O-6 of every third backbone residue. Viscometry and Congo red reaction indicated that HEP3 has a highly ordered hydrogen-bond dependent conformation in aqueous solution, which collapses in strong alkaline solution.

Keywords: *Hericium erinaceus*; β-(1→3)-D-Glucan; Chemical structure; Conformation

Hericium erinaceus (Bull. ex Fr.) Pers (lion's mane) is an edible basidiomycetous fungus (Hydnaceae). Its fruiting bodies are well known as a traditional Chinese medicine or food and have attracted much investigation due to their health effects when used as a home remedy for gastric and duodenal ulcers and some other diseases.[1] This fungus contains polysaccharides, which exhibit immunomodulating activity and antiradiative effects.[2,3] A rhamnoglucogalactan fraction has been isolated from the boiling water extract and structurally characterized.[4] As we know, branched (1→3)-β-D-glucans are common components of the cell wall of many basidiomycetous fungi and are often reported to show immunomodulatory activity.[5,6] In this communication, we describe a branched (1→3)-β-D-glucan, designated HEP3, isolated from the fruiting bodies of *H. erinaceus*. Its aqueous conformation is revealed by the viscometric method and complex formation with Congo red in alkaline solution.

HEP3 was obtained from the fruiting bodies of *H. erinaceus* in a yield of ca. 0.2% on the basis of the crude material, using alkali extraction and anion-exchange chromatography on a DEAE-cellulose column. On HPGPC, HEP3 showed a symmetrical peak, indicating a homogenous fraction. The average molecular weight was estimated to be higher than 1.0×10^6 g/mol. After complete hydrolysis with 2 M trifluoroacetic acid (TFA), TLC analysis showed that the polysaccharide contains no uronic acid. GLC analysis indicated that it was composed exclusively of glucose. The absorption at 890 cm^{-1} in the IR indicated that HEP3 has β-glucopyranosidic linkages, which was further supported by its low $[\alpha]_{20}^{D}$ value of +14.6 (*c* 0.29, H_2O).

The ^1H NMR spectrum (not shown) of HEP3 displayed only one anomeric signal at δ 4.6 ppm, and the ^{13}C NMR spectrum contained two anomeric signals at δ 103.46 ppm and δ 102.97 ppm, further indicating a β-anomeric configuration for glucopyranosyl units.[7] The signals at δ 86.72 and δ 86.0 ppm arose from the substituted C-3 of glucose. The signal at δ 69.02 ppm was assigned to C-6 of branched (1→3)-β-D-glucosyl residues, as was supported by the corresponding

* Corresponding author. Tel.: +86 21 5080 6600; fax: +86 21 5080 7088; e-mail: dongqun@hotmail.com

0008-6215/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2006.01.022

Table 1. ^{13}C NMR spectral assignments of HEP3 in D_2O–NaOD[a]

Residues	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*(1→	102.97	75.00	76.11	70.04	76.11	60.96
→3)-β-D-Glc*p*(1→	103.46	73.60	86.72	68.42	76.11	60.96
→3,6)-β-D-Glc*p*(1→	103.46	73.60	86.00	68.42	75.00	69.02

[a] Concentration, 30 mg/0.5 mL.

Table 2. GC–MS data for methylation analysis of HEP3

Methylated sugars	Linkage types	Molar ratios (mol %)	Major mass fragments (*m*/*z*)
2,3,4,6-Me_4-Glc	Terminal	23.9	45, 71, 87, 101, 117, 129, 145, 161, 205
2,4,6-Me_3-Glc	1,3-	50.5	45, 58, 71, 87, 99, 101, 117, 129, 161, 201, 233
2,4-Me_2-Glc	1,3,6-	25.6	58, 87, 99, 101, 117, 129, 139, 159, 189, 201, 233

reversed peak in the DEPT spectrum (not shown). Other ^{13}C NMR signals were tentatively assigned and are shown in Table 1, referred to the literature values.[8]

After methylating three times using the modified Ciucanu method, the OH absorption at 3600–3200 cm^{-1} in IR disappeared, indicating the completeness of methylation. The permethylated polysaccharide was depolymerized and converted into partially methylated alditol acetates. GC–MS analysis showed three types of linkages, corresponding to T-Glc*p* (terminal), 1,3-linked Glc*p* and 1,3,6-linked Glc*p*, respectively, approximately in the molar ratio of 1:2:1. The data indicated an O-6-branched (1→3)-β-D-glucan structure of HEP3 (Table 2).

In order to ascertain whether HEP3 has a single unit D-glucosyl or (1→3)-linked multiple unit side chains, periodate oxidation and Smith degradation were carried out. After 5 days of periodate oxidation, HEP3 consumed 0.5 mol of periodate and produced 0.27 mol of formic acid per mole of glucosyl residues. This result was in good accord with the theoretical values (0.5 mol of periodate and 0.25 mol of formic acid) calculated from methylation analysis. HEP3-SA, the polyol derivative from periodate oxidation, was composed of D-glucose and glycerol in a molar ratio of 2.9:1, also in agreement with the data above.

HEP3-SP, the Smith-degradation product of HEP3, showed six ^{13}C NMR signals, which were assigned clearly to the six carbons of (1→3)-linked β-D-glucosyl units: δ 103.37 ppm (C1), 73.54 ppm (C2), 86.61 ppm (C3), 68.55 ppm (C4), 76.42 ppm (C5), 61.00 ppm (C6). The disappearance of terminal and (1→3)- and (1→6)-linked Glc*p* in its linkage analysis further confirmed a linear structure for HEP3-SP. These results suggested that HEP3 contains branches of a single glucosyl unit, which were completely removed by Smith degradation (Fig. 1).

It could thus be concluded that HEP3 has a backbone of (1→3)-linked β-D-glucopyranosyl units, with one single unit β-D-glucopyranosyl branch substituted at O-6,

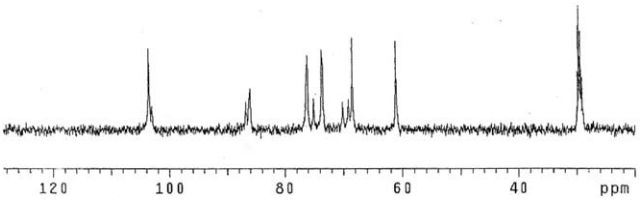

Figure 1. ^{13}C NMR spectrum of polysaccharide HEP3 isolated from *Hericium erinaceus*. The sample was dissolved in D_2O–NaOD (30 mg/0.5 mL) and determined at 30 °C, with acetone as the internal standard (δ 29.50 ppm).

on average, for every three backbone units (Fig. 2). This result was in agreement with other same types of β-D-glucans isolated from other fungi or lichens.[8–10]

It has been reported that many (1→3)-(1→6)-β-D-glucans adopt ordered helical conformations in aqueous solution.[11,12] A strong alkaline environment can induce the denaturation of such helical structures by breaking the intra- and intermolecular hydrogen bonds, leading to reduced aqueous viscosity.[13] As shown in Figure 3, significant reduction of intrinsic viscosity [η] was observed as NaOH concentration increases from 0 to 0.2 M, reflecting a continuous denaturation process of

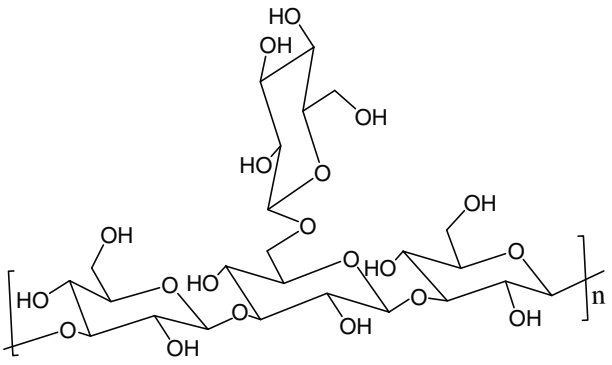

Figure 2. The proposed structure for the native polysaccharide, HEP3.

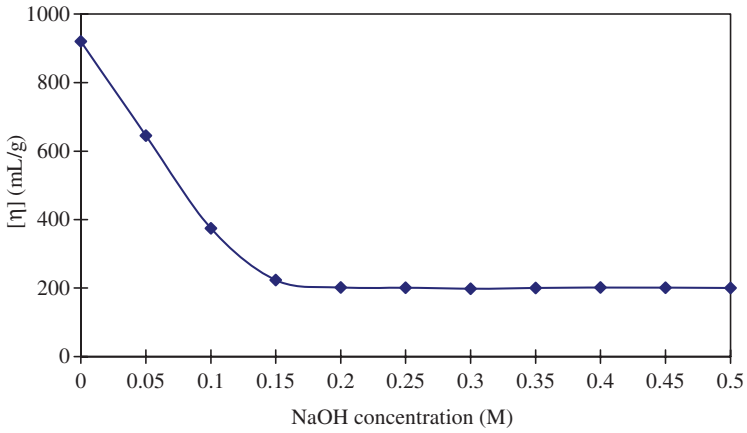

Figure 3. The change of intrinsic viscosity [η] (mL/g) of HEP3 at different NaOH concentrations at 24 ± 0.5 °C. HEP3 concn: 0.5 mg/mL.

the highly ordered conformation of HEP3. The change of intrinsic viscosity indicated that the hydrogen-bonding structure broke down gradually with the addition of NaOH and was destroyed completely when the NaOH concentration reaches 0.2 M.

(1→3)-β-D-Glucans with a helical conformation have been reported to form complexes with Congo red in a dilute alkaline solution as indicated by a shift in the maximum absorption wavelength of Congo red (λ_{max}).[14] Figure 4 shows the shift of λ_{max} in different concentrations of NaOH in the presence of HEP3 and some λ_{max}s of Congo red complexes with HEP3 or HEP3-SP are given in Table 3. At low NaOH concentrations (0.1–0.15 M), the λ_{max} shifted to a longer wavelength and reached the longest wavelength (512 nm) at the alkaline concentration of about 0.11 M. When the NaOH

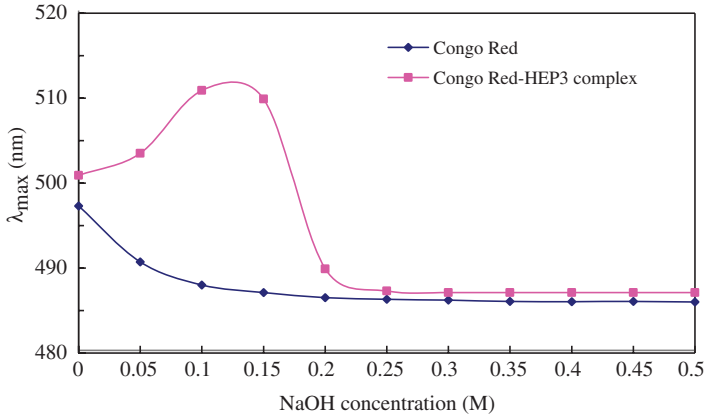

Figure 4. Change in λ_{max} of Congo red and Congo red–HEP3 complex at different NaOH concentrations. The solutions contain 24.4 μM of Congo red and 0.5 mg/mL of HEP3.

Table 3. λ_{max}s of Congo red complexed with HEP3 or HEP3-SP in different NaOH solutions

Fractions	c (NaOH)[a]	λ_{max} (nm)	λ_{max} (nm) without glucan[b]	λ_{max} shift[c] (nm)
HEP3	0	500.6	497.0	3.6
	0.1 M	510.6	487.7	22.9
	0.5 M	487.9	486.8	1.1
	0.5 M, then neutralized	511.3	497.0	14.3
HEP3-SP	0	503.0	497.0	6.0
	0.1 M	514.4	487.7	26.7
	0.5 M	504.6	486.8	18.9

[a] Concentration of NaOH.
[b] The λ_{max} of Congo red was determined at the same NaOH concentration as for Congo red complex.
[c] The λ_{max} shifts were calculated by subtracting the λ_{max} of Congo red from that of Congo red complex.

concentration is higher than 0.15 M, the λ_{max} dropped sharply and the curve flattened at 486.8 nm when NaOH concentrations are higher than 0.25 M. After neutralization with 2 M HCl, the λ_{max} in 0.5 M NaOH concentration shifted from 486 to 502 nm, which was much close to the value in a neutral solution (500.6 nm). The shift of λ_{max} indicated that the secondary structure of HEP3 dissociated completely at a NaOH concentration higher than 0.25 M and that this denaturation could be reversed by neutralization. Furthermore, for HEP3-SP, the λ_{max} of Congo red shifted to 514.4 nm in 0.1 M NaOH and almost kept stable at 504.6 nm even in 0.5 M NaOH, probably suggesting that HEP3-SP adopted a highly ordered conformation, which remained stable even under strong alkaline conditions.

Both the studies on intrinsic viscosity and the Congo red–HEP3 complex indicated that HEP3 adopts an ordered hydrogen-bond dependent conformation in neutral and slightly alkaline aqueous solution, which was denatured under strong alkaline condition ($c_{NaOH} >$ 0.15 M), probably transformed into a random coil.

1. Experimental

1.1. Materials and general methods

The fruiting bodies of *H. erinaceus* (Bull. ex Fr.) Pers were cultivated, collected in *Zhejiang* Province, China, and obtained as dried crude drug from Qingyuan Fangge Pharmaceutical Co. DEAE-Cellulose 32 was from Whatman Co. The optical rotation was determined with a WZZ-1S automatic polarimeter (Shanghai Physical Optics Instruments Co.). IR spectra were determined using a Perkin–Elmer 591B spectrophotometer with a KBr pellet (native polysaccharide) or Nujol film (permethylated polysaccharide). GLC analyses were carried out with a Shimadzu-14B apparatus equipped with a 3% OV-225/AW-DMCS-Chromosorb W column (2.5 m × 3 mm) and an FID detector.

1.2. Isolation of polysaccharides

Fruiting bodies (1.5 kg) of *H. erinaceus* were defatted by refluxing with 95% EtOH in a Soxhlet extractor, and the residue was extracted successively with boiling water (6 h) for three times. The water-insoluble residues were then extracted twice with 1 M NaOH at 4 °C (each for 4 h). The filtrate from the alkaline extraction was neutralized with 3 M HCl at room temperature, dialyzed, concentrated and centrifuged. Addition of 95% EtOH (3 vol) to the supernatant yielded a water-soluble crude polysaccharide, CPB1 (19.7 g), as the precipitate. Redissolved in water (1%), 10 g of CPB1 was fractionated on a DEAE-cellulose (Cl^-) column by sequential elution with water, and then by 0.1, 0.2 and 0.4 M NaCl solutions. The water-eluted fraction was further fractionated on a DEAE-cellulose (OH^-) column, which was eluted with water, followed by 0.01, 0.02 and 0.04 M NaOH. The last eluted fraction was collected according to the sugar profile detected by phenol–H_2SO_4 method, concentrated, neutralized with HOAc, dialyzed and lyophilized to give HEP3 (1.4 g).

1.3. Homogeneity and molecular weight

High-performance gel permeation chromatography (HPGPC) was carried out with a Waters 515 pump equipped with a Waters Ultrahydrogel™ 1000 column and a Waters 2410 RI detector. The column was calibrated with standard T-series Dextran (T-500, T-110, T-80, T-70, T-40 and T-9.3) with 0.003 M NaOAc as the mobile phase at a flow rate of 0.5 mL/min. All samples were prepared as 0.4% (w/v) solutions and 20 μL of solution was analyzed in each run. The data were processed with Waters GPC Millennium[32] software.

1.4. Monosaccharide analysis and linkage analysis[15]

The polysaccharide (3 mg) was hydrolyzed with 2 M TFA at 110 °C for 3 h, followed by evaporation to dryness. The residue was redissolved in water (0.2 mL), with 5 μL of the solution used for TLC analysis as described previously. The other portion was successively reduced with sodium borohydride, acetylated with Ac_2O at 100 °C for 1 h, and the resulting alditol acetates were examined by GLC.

The purified polysaccharide was methylated three times using the modified Ciucanu method.[16] The permethylated polysaccharide was depolymerized with 90% formic acid (100 °C, 4 h), followed by hydrolysis with 2 M TFA (100 °C, 6 h). The hydrolysate was converted into partially methylated alditol acetates and analyzed by GC–MS with a Shimadzu QP Class-5000 instrument.

1.5. Periodate oxidation and Smith degradation

The polysaccharide (200 mg) was oxidized in 0.015 M $NaIO_4$ (200 mL) at 5 °C in the dark (5 days) and monitored spectrophotometrically. The periodate consumption was calculated from the change of absorption at 223 nm.[17] The formic acid production was measured by titration with 0.01 M NaOH. After complete oxidation, 2 mL of ethylene glycol was added, and the reaction mixture was dialyzed. The resulting polyaldehydes was reduced with $NaBH_4$ (0.5 g) for 18 h, then neutralized with HOAc, dialyzed and lyophilized to produce the polyol derivative, HEP3-SA (149 mg). A portion of HEP3-SA (60 mg) was hydrolyzed with 0.1 M TFA at 40 °C for 24 h, then dialyzed, concentrated and lyophi-

lized, giving the Smith degradation product, HEP3-SP (39 mg).

1.6. ^{1}H NMR and ^{13}C NMR spectroscopy

^{1}H and ^{13}C NMR spectra were measured using a Brüker AM-400 NMR instrument equipped with a dual probe in the FT mode at room temperature. The polysaccharides HEP3 and HEP3-SP were dissolved in D_2O–NaOD at a concentration of 30 mg/0.5 mL. Acetone was used as the internal standard (δ 29.47 ppm). All chemical shifts are expressed in reference to tetramethylsilane.

1.7. Intrinsic viscosities ($[\eta]$) and Congo red assay

The solutions of HEP3 (0.5 mg/mL) in 0–0.5 M NaOH (with a stepwise increment of 0.05 M in NaOH concentration) were prepared, and their intrinsic viscosities $[\eta]$ were determined with a Ubbelohde type viscometer at 24 ± 0.5 °C. The kinetic energy correction was negligible. $[\eta]$ was estimated using the Huggins and Kraemer equations by extrapolation to infinite dilution as follows:

$$\eta_{sp}/c = [\eta] + k'[\eta]^2 c$$
$$(\ln \eta_r)/c = [\eta] - k''[\eta]^2 c$$

where k' and k'' are constants for a given polymer under given conditions in a given solvent; η_{sp}/c the reduced specific viscosity; $(\ln \eta_r)/c$ the inherent viscosity.

For Congo red reaction, HEP3 solutions were prepared in the same way as described above for viscosity determination except containing 24.4 μM of Congo red. The absorption spectrum was recorded from 400 to 700 nm at 25 °C with a UV-260 Shimadzu spectrophotometer. In addition, the λ_{max}s of Congo red in HEP3-SP solution at 0.1 and 0.5 M NaOH concentration were also measured.

References

1. Traditional Chinese Medicine Editorial Committee, State Traditional Chinese Medicine Administration. In *Chinese Medicinal Herbs*; Shanghai Science and Technology Publisher: Shanghai, 1999.
2. Xu, H. M.; Xie, Z. H.; Zhang, W. Y.; Jiang, W. H. *Chin. J. Integrat. Tradit. West. Med.* **1994**, *14*, 427–428.
3. Liu, S. C.; Zhang, H. J.; Lao, C. H.; Wang, B. *Chin. J. Radiol. Med. Prot.* **1999**, *19*, 328–329.
4. Jia, L. M.; Liu, L.; Dong, Q.; Fang, J. N. *Carbohydr. Res.* **2004**, *339*, 2667–2671.
5. Bao, X. F.; Liu, C. P.; Fang, J. N.; Li, X. Y. *Carbohydr. Res.* **2001**, *332*, 67–74.
6. Molinaro, A.; Lanzetta, R.; Mancino, A. *Carbohydr. Res.* **2000**, *329*, 441–445.
7. Perret, J.; Brunneteau, M.; Michel, G.; Marais, M. F.; Joseleau, J. P.; Ricci, P. *Carbohydr. Polym.* **1992**, *17*, 231–236.
8. Barbosa, A.; Steluti, R. M.; Dekker, R. F. H.; Cardoso, M. S.; da Silva, M. L. C. *Carbohydr. Res.* **2003**, *338*, 1691–1698.
9. Deng, C.; Yang, X.; Gu, X.; Wang, Y.; Zhou, J.; Xu, H. *Carbohydr. Res.* **2000**, *328*, 629–633.
10. Olafsdottir, E. S.; Omarsdotti, S.; Paulsen, B. S.; Wagner, H. *Phytomedicine* **2003**, *10*, 318–324.
11. Falch, B. H.; Espevik, T.; Ryan, L.; Stokke, B. T. *Carbohydr. Res.* **2000**, *329*, 587–596.
12. Sletmoen, M.; Christensen, B. E.; Stokke, B. T. *Carbohydr. Res.* **2005**, *340*, 971–979.
13. Zhang, P. Y.; Zhang, L. N.; Cheng, S. Y. *Carbohydr. Res.* **2000**, *327*, 431–438.
14. Ohno, N.; Yadomae, T. *Carbohydr. Res.* **1987**, *159*, 293–302.
15. Dong, Q.; Fang, J. N. *Carbohydr. Res.* **2001**, *332*, 109–114.
16. Needs, P. W.; Selvendran, R. R. *Carbohydr. Res.* **1993**, *245*, 1–10.
17. Dixon, J. S.; Lipkin, D. *Anal. Chem.* **1954**, *26*, 1092–1093.