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Note

The structure and conformation of a water-insoluble (1→3)-,(1→6)-β-D-glucan from the fruiting bodies of *Pleurotus florida*

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Abstract—A water-insoluble glucan, PFPSIN, has been isolated from the aqueous extract of an edible mushroom *Pleurotus florida*. On the basis of total acid hydrolysis, methylation analysis, periodate oxidation, Smith degradation, and ^{13}C NMR experiments, the repeating unit of the polysaccharide was established as

→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→
6
↑
1
β-D-Glc*p*

Conformational analysis revealed the triple helical conformation of this glucan.

Keywords: Insoluble glucan; *Pleurotus florida*; Triple helix; Conformation

Several water-insoluble (1→3)-,(1→6)-β-D-glucans[1–3] isolated from different mushrooms are well known for their immunomodulatory[4,5] and antitumor properties.[6,7] A β-glucan may exist in either single helical[8,9] or triple helical conformation.[10] Therefore, attention is being devoted to the conformational behavior of (1→3)-, (1→6)-β-D-glucans.

Pleurotus florida is a delicious edible mushroom with high therapeutic value. Three different water-soluble polysaccharides were isolated from this mushroom by our group and have already been reported.[11–13] In addition, there is another water-insoluble polysaccharide, named PFPSIN, that has been isolated from the aqueous extract of *Pleurotus florida* and a detailed structural analysis of this polysaccharide is reported here.

PFPSIN was obtained from *P. florida* by hot water extraction, precipitation in ethanol, fractionation with 1% NaCl solution, and then alkali treatment. The polysaccharide was hydrolyzed with 2 M trifluoroacetic acid. The analysis of alditol acetates by PC and GLC showed the presence of only glucose. The absorption at 890 cm^{-1} in the IR spectrum indicated that PFPSIN has β-glucopyranosidic linkages,[14] which was further supported by its low $[\alpha]_{25}$ +18.8. The absolute configuration[15] of glucose was determined as D. The glucan was methylated according to the method of Ciucanu and Kerek,[16] and GLC and GLC–MS analyses of alditol acetates obtained from the hydrolyzate of the methylated polysaccharide revealed the presence of 1,5-di-*O*-acetyl-2, 3,4,6-tetra-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4, 6-tri-*O*-methyl-D-glucitol, and 1,3,5,6-tetra-*O*-acetyl-2, 4-di-*O*-methyl-D-glucitol in a ratio of nearly 1:2:1, respectively (Table 1, Fig. 1). These results indicate the presence of nonreducing end D-glucopyranosyl, (1→3)-linked D-glucopyranosyl, and (1→3, 1→6)-linked D-glucopyranosyl (branch point) moieties in the glucan. Furthermore, GLC–MS analysis of the alditol acetates of the periodate-oxidized, reduced, methylated polysaccharide

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Table 1. GLC–MS data for the methylated sugar moieties of PFPSIN isolated from *P. florida*

Methylated sugars	Linkage types	Molar ratio	Major fragments (*m*/*z*)
2,3,4,6-Me_4-Glc*p*	Terminal	1	45, 71, 87, 101, 117, 129, 145, 161, 205
2,4,6-Me_3-Glc*p*	→3)-Glc*p*-(1→	2	45, 58, 71, 87, 99, 101, 117, 129, 143, 161, 201, 233
2,4-Me_2-Glc*p*	→3,6)-Glc*p*-(1→	1	58, 87, 99, 101, 117, 129, 139, 159, 189, 233

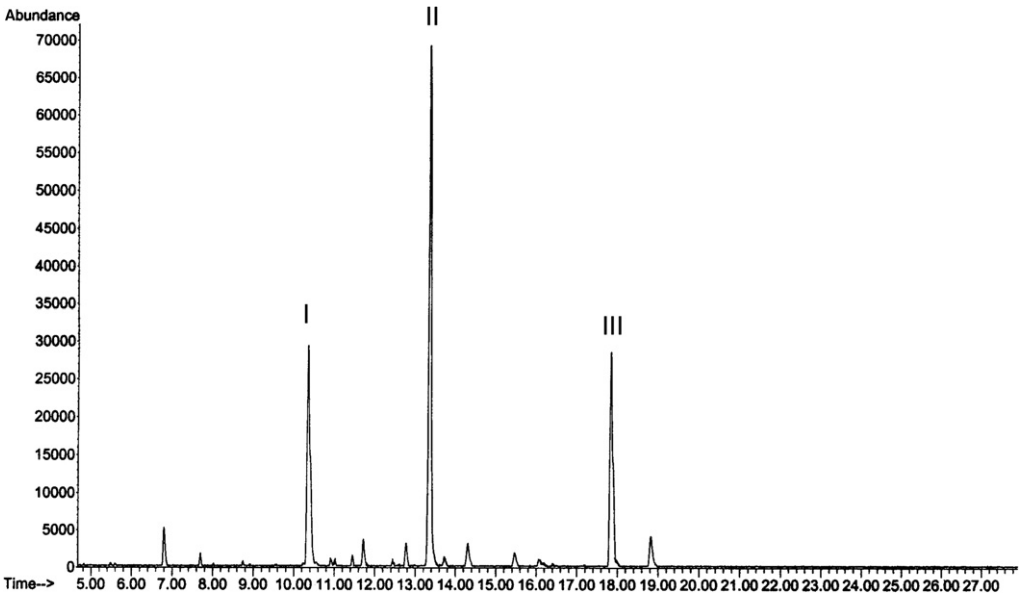

Figure 1. GLC of the methylated alditol acetates derived from the polysaccharide (PFPSIN) isolated from *P. florida* using a HP-5 fused silica capillary column.

showed the presence of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4, 6-tri-*O*-methyl-D-glucitol in a ratio of nearly 1:2. The absence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol indicates that the nonreducing end D-glucopyranosyl moiety is consumed during oxidation.

To ascertain whether PFPSIN has a single D-glucosyl unit or (1→3)-linked multiple unit D-glucosyl side chains, a Smith degradation[17] experiment was carried out with this glucan. The degraded glucan was methylated, and the alditol acetates on GLC and GLC–MS analyses revealed the presence of only 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol. This result further confirms that the nonreducing end D-glucopyranosyl residue attached to the branching point at C-6 of glucan is completely consumed during oxidation and a linear (1→3)-linked D-glucan is produced. The disappearance of terminal and (1→3,6)-linked D-glucopyranosyl moieties in linkage analysis of Smith degraded product suggests that PFPSIN contains branches of a single glucosyl unit, which were completely removed during Smith degradation reaction.

A ^{13}C NMR (100 MHz) spectrum of the polysaccharide was obtained on a Me_2SO-d_6 solution of PFPSIN because the β-D-glucan is insoluble in water. This spectrum (Fig. 2) shows multiple resonances, which agree with the branched (1→3),(1→6)-β-D-glucan structure. The β-configuration of D-glucosyl residues is clearly evidenced by the presence of an anomeric peak at δ 103.34 ppm. The signal at δ 69.54 ppm is assigned to C-6 of branched (1→3,6)-β-D-glucosyl residue. The downfield chemical shift of the substituted C-6 compared to the unsubstituted C-6 of methyl glycosides is due to the α-effect of glycosylation. The signals at δ 87.27 ppm and δ 86.67 ppm arise from C-3 of (1→3)-linked β-D-glucosyl and (1→3,6)-β-D-glucosyl residues, respectively. The C-3 signals are shifted downfield compared to the resonance of standard methyl glycosides due to the α-effect of glycosylation.[18] The multiplicity of the signals and also C-3 signals could be ascribed to the presence of linear (1→3)-β-D-, and branched (1→3,6)-β-D-glucopyranosyl residues. The ^{13}C NMR signals were tentatively assigned and are shown in Table 2. From the above experimental results it thus could be concluded that PFPSIN has a backbone of (1→3)-linked β-D-glucopyranosyl units, with one single unit β-D-glucopyranosyl branch substituted at O-6 for every three-backbone units (Fig. 3). This result was in agreement with other types of β-D-glucans isolated from other fungi and lichens.[19–22]

The conformational behavior of the branched (1→3),(1→6)-β-D-glucan has been discussed in regard to changes in specific rotation, and in the visible absorption spectra of the complexes formed with Congo red, at

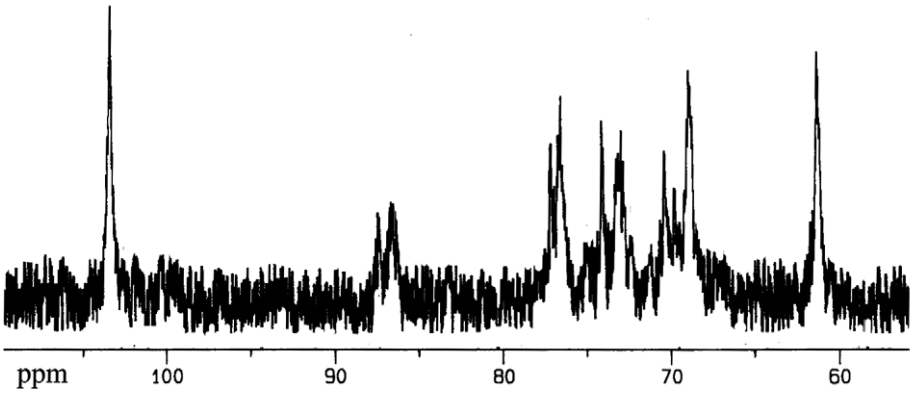

Figure 2. ^{13}C NMR (100 MHz, Me_2SO-d_6, 27 °C) spectrum of the polysaccharide (PFPSIN) isolated from *P. florida.*

Table 2. The chemical shifts of ^{13}C NMR of PFPSIN[a] in Me_2SO-d_6

Residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*	103.34	74.13	77.13	70.40	76.55	61.32
→3)-β-D-Glc*p*-(1→	103.34	72.98	87.27	68.96	76.55	61.32
→3,6)-β-D-Glc*p*-(1→	103.34	72.98	86.67	68.96	74.13	69.54

[a] Values of chemical shifts were recorded with reference to acetone as internal standard and fixed at δ 30.05 ppm at 27 °C.

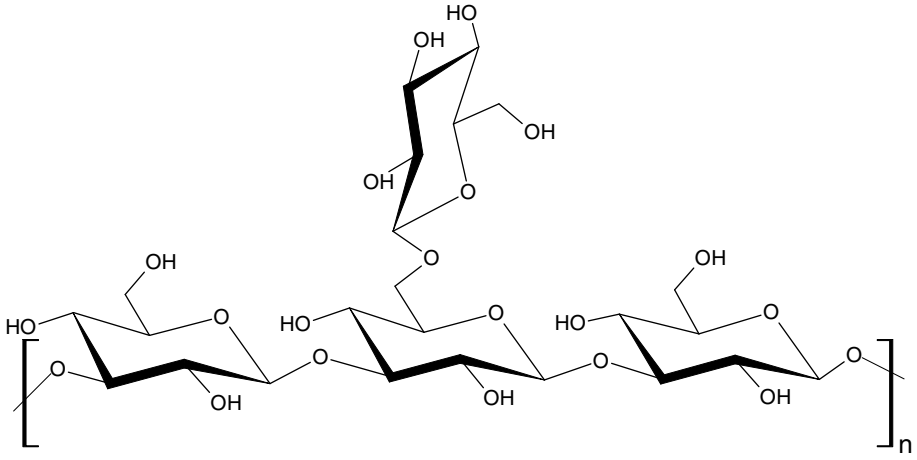

Figure 3. The proposed structure for the native polysaccharide (PFPSIN) isolated from *P. florida.*

various concentrations of alkali. The value of specific rotations of PFPSIN was decreased at alkaline concentrations in the range of 0.15–0.30 M (Fig. 4). The complex formation of PFPSIN with Congo red was evaluated from the shift in the visible absorption maximum (λ_{max}) of Congo red at various concentrations of sodium hydroxide according to Ogawa et al.[23] As shown in Figure 5, the values of λ_{max} of Congo red are largely shifted by the presence of PFPSIN to a longer wavelength (λ_{max} 500–514 nm) in 0.1 M NaOH solution[10] and the λ_{max} is reduced and the curve is flattened at 506 nm and remains the same even in high concentrations of alkali. This suggests that the conformational structure of the glucan (PFPSIN) has been lost at concentrations of 0.3 M NaOH and higher. Thus, from the present study, it can be concluded that PFPSIN has triple helical structure. Some linear and branched (1→3)-β-D-glucans, for example, curdlan (isolated from *Alcaligenes faecalis*[8]), Schizophyllan,[24] and T-N-5[10] have been reported to have a triple helical conformation in aqueous or weakly alkaline solution (<0.15 M NaOH), which is transformed into single chains in highly alkaline solutions (>0.25 M NaOH).

1. Experimental

1.1. Isolation and purification

Two kilograms of fresh mushroom fruit bodies was collected from local firm where it was grown on paddy straw at a temperature of 28 °C. After washing with dis-

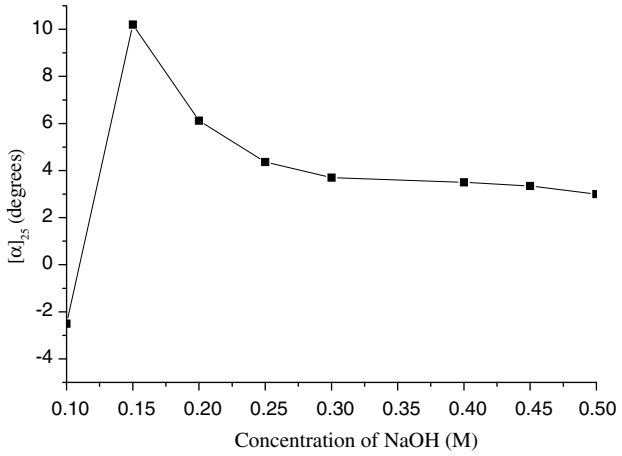

Figure 4. Dependence of the specific rotation of PFPSIN, at 589 nm on the different concentrations of sodium hydroxide solution.

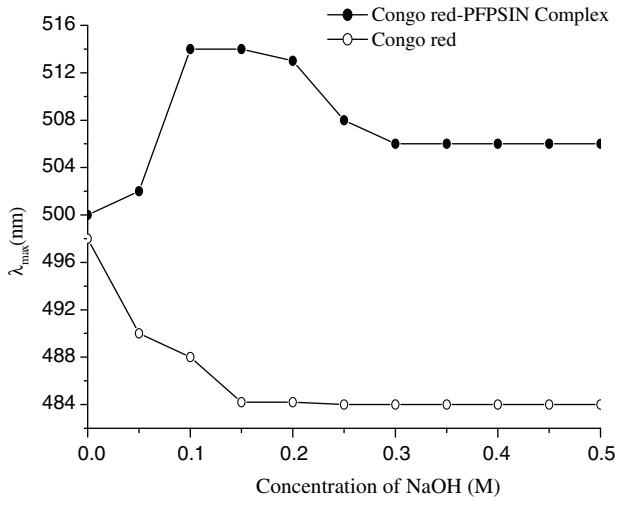

Figure 5. Change in the absorption maximum of the Congo red-PFPSIN complex at various concentrations of sodium hydroxide solution.

tilled water and ethanol, the mushrooms were pulverized for the extraction of polysaccharide by boiling with water for 4 h. The mixtures were filtered and the filtrate was centrifuged at 8000 rpm at 6 °C for 45 min to obtain clear solution, and the polysaccharide was precipitated with ethanol (80%, final concentration). After keeping the precipitate at 4 °C overnight, it was separated by centrifugation at low temperature and again solubilized with water and then dialyzed against distilled water. After re-precipitation with ethanol, the precipitate was separated by centrifugation and then freeze-dried (yield, 2 g).

The freeze-dried polysaccharide was found to be sparingly soluble in water. The water-insoluble glucan was isolated from the whole aqueous extract by dissolving it in 1% NaCl solution. The insoluble portion in 1% NaCl solution was separated by centrifugation at 6000 rpm for 30 min. The water was added to the insoluble portion and dialyzed against water to remove NaCl from insoluble portion. The water-insoluble portion was then dissolved in 4 M NaOH solution. The insoluble material was removed by centrifugation. The filtrate was collected and then neutralized with acetic acid. The polysaccharide was precipitated in EtOH. The precipitate was collected by centrifugation and then freeze-dried. The carbohydrate content of the insoluble fraction was determined using phenol–sulfuric acid method.[25]

1.2. Monosaccharide composition

The polysaccharide (3 mg) with 2 M trifluoroacetic acid (2 mL) was hydrolyzed in a boiling water bath for 16 h and the acid was removed by co-distillation with water. The monosaccharides were reduced by $NaBH_4$, followed by acidification with acetic acid to alditols. The alditols were then converted into alditol acetates by heating with (1:1) pyridine–Ac_2O for 30 min at 100 °C and analyzed by GLC performed with a Hewlett–Packard model 5730 gas chromatograph equipped with a flame-ionization detector. The instrument was fitted with a glass column (1.8 m × 6 mm) packed with 3% ECNSS-M (A) on Gaschrom-Q (100–120 mesh) and 1% OV-225 (B) on Gaschrom-Q (100–120 mesh). Quantitation was carried out from peak area, using response factors from standard monosaccharide.

1.3. Absolute configuration of monosaccharides

The method used was based on Gerwig et al.[15] After trifluoroacetic acid hydrolysis of 1 mg of polysaccharide, the acid was removed, 250 μL of 0.625 M HCl in R-(+)-2-butanol was added and after 16 h at 80 °C, the reactant was evaporated and TMS-derivatives were prepared with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The products were analyzed by GLC using a capillary column SPB-1 (30 m × 0.26 mm), a temperature program (3 °C/min) from 150 to 210 °C. The product 2,3,4,6-tetra-*O*-TMS-(+)-2-butyl glycosides were identified by comparison with those prepared from the D and L enantiomers of different monosaccharides.

1.4. Methylation analysis

The polysaccharide (4 mg) was methylated according to the method of Ciucanu and Kerek,[16] and the products were isolated by partition between chloroform and water (5:2). The organic layer containing products was washed with water three times and dried. Then, methylated products were hydrolyzed by treatment with 90% formic acid (100 °C, 1 h), and the monosaccharides were converted to their methylated alditol acetates as usual.

The sugar linkages of the constituent methylated alditol acetates were analyzed by GLC using columns A and B as above and also by GLC–MS analysis, performed on a Hewlett–Packard 5988A automatic GLC–MS system with an HP-5 fused silica capillary column using a temperature program from 150 °C (2 min) to 200 °C (5 min) at 2 °C min^{-1}.

1.5. Periodate oxidation

The polysaccharide (4.0 mg) in 0.1 M $NaIO_4$ (2 mL) was kept in the dark for 48 h at room temperature. Excess sodium metaperiodate was destroyed by the addition of 1,2-ethanediol and the mixture was dialyzed against distilled water. The product was reduced overnight with $NaBH_4$, neutralized with acetic acid, and dried by the addition of MeOH. The periodate-treated polysaccharide was then hydrolyzed with 2 M CF_3COOH for 16 h, and the alditol acetates were prepared and analyzed by GLC using columns A and B.

1.6. Smith degradation[17]

The polysaccharide was oxidized with 0.1 M sodium-metaperiodate (10 mL) at 27 °C in the dark for 48 h. The oxidation was stopped by the addition of 1,2-ethanediol and the solution was dialyzed against distilled H_2O. The dialyzed material was reduced with $NaBH_4$ for 15 h at 27 °C, neutralized with 50% HOAc and again dialyzed against distilled water, and freeze-dried. This portion was subjected to mild hydrolysis with 0.5 M trifluoroacetic acid for 15 h at 25 °C to eliminate the residue of oxidized sugars attached to the polysaccharide chain (Smith degradation). The acid was removed after repeated addition and evaporation of water at 37 °C. Finally, it was purified by exhaustive dialysis against distilled water and freeze-dried. This polymeric material (2 mg) was methylated and analyzed as usual by GLC–MS.

1.7. NMR spectroscopy

The ^{13}C NMR experiment was recorded at 100 MHz on a Bruker Avance DPX-400 spectrometer using Me_2SO-d_6 as solvent at 27 °C. Acetone was used as an internal standard (δ 31.01 ppm).

1.8. Interaction with Congo red

The interaction with Congo red was evaluated from the shift in the visible absorption maximum of Congo red that was induced by the presence of polysaccharide at various concentrations of alkali, according to the method of Ogawa et al.[23] The solutions of PFPSIN (0.5 mg/mL) in 0–0.5 M NaOH containing 91 μM of Congo red were prepared. The absorption spectra were recorded from 400 to 700 nm at room temperature with a UV-1601 Shimadzu spectrophotometer.

1.9. Specific rotations in aqueous sodium hydroxide

The polysaccharide (15 mg) was dissolved in water (10 mL), and the concentration of alkali was increased from 0 to 0.5 M by the stepwise addition of 4 M NaOH solution. Specific rotations were measured with a Jasco P 1020 automatic polarimeter at 25 °C at each concentration of alkali.

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